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(71) Applicant: THE CENTER FOR BLOOD RESEARCH [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US).

(72) Inventors: STAUNTON, Donald, E. ; 124 Chestnut Hill Road, Chestnut Hill, MA 02167 (US). SPRINGER, Timothy, A. ; 28 Monadnock Road, Chestnut Hill, MA 02167 (US).

(74) Agents: FOX, Samuel, L. et al.; Sterne, Kessler, Goldstein & Fox, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).

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(54) Title: CD36 IMMUNOADHESINS AND THEIR USE IN SELECTIVELY KILLING *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES

(57) Abstract

The present invention discloses the construction of CD36 immunoadhesin and their use in selectively killing *Plasmodium Falciparum* infected erythrocytes. Disclosed are immunoadhesin containing CD36 or fragments of CD36 deleted for one or more of the regions of residues 1-5, 6-28, 439-465 and 466-471.

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# CD36 IMMUNOADHESINS AND THEIR USE IN SELECTIVELY KILLING *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES

This application is a continuation-in-part of U.S. Serial No. 07/862,708 filed April 3, 1992, which is a continuation-in-part of U.S. Serial No. 07/769,625 filed October 3, 1991.

## FIELD OF THE INVENTION

The present invention relates to agents which bind to the ICAM-1 or the CD36 binding site on malarially infected erythrocytes (IRBC). The present invention additionally relates to molecules capable of binding to the IRBC binding site on ICAM-1 or on CD36. The agents of the present invention include antibodies, peptides, and carbohydrates. These agents are useful in ameliorating the symptoms of malaria since they are capable of inhibiting the binding of an IRBC to either ICAM-1 or CD36 and stimulating the phagocytosis of IRBCs.

The present invention further provides methods for the treatment of malaria, methods of preferentially killing an IRBC, methods of stimulating phagocytosis of an IRBC, and a method of diagnosing the presence of an IRBC.

## BACKGROUND OF THE INVENTION

### Malaria

Erythrocytes infected with the human malaria parasite, *Plasmodium falciparum*, adhere to vascular post-capillary endothelium, and the sequestration of the malaria-infected erythrocytes (IRBC) is a primary event responsible for the clinical complications of severe and cerebral malaria. While immature ring stage parasitized erythrocytes circulate unobstructed throughout the vasculature, adhesion of mature intraerythrocytic stages of the parasite to endothelium averts splenic clearance of IRBC and allows parasite maturation in a microenvironment of low oxygen tension. Two cell surface receptors with broad tissue distribution, intercellular adhesion molecule-1 (ICAM-1, CD54) (Berendt *et al.*, *Nature (Lond.) 341*:57-59 (1989)) and CD36 (GPIV) (Ockenhouse *et al.*, *Science (Wash. D.C.) 243*:1469-1471 (1989)) have recently been identified as endothelial receptors for IRBC. Laboratory-adapted IRBC bind to purified ICAM-1-coated and CD36-coated surfaces and the cytoadherent phenotype of these malaria-infected red cells can be modulated by successive panning on ICAM-1 or CD36-coated surfaces (Ockenhouse *et al.*, *J. Infect. Dis. 164*:163-169 (1991)). Moreover, ICAM-1-specific and CD36-specific monoclonal antibody (MAb) staining of small capillary endothelium from postmortem brain tissue colocalizes with IRBC cytoadherence in patients who have died from complications of cerebral malaria (Barnwell *et al.*, *J. Clin. Invest. 84*:765-772 (1989); Aikawa *et al.*, *Am. J. Trop. Med. Hyg. 43*:30 (1990)).

ICAM-1

ICAM-1, a member of the immunoglobulin-like superfamily, is a monomeric unpaired 90-115 $M_r$ glycoprotein composed of a bent extracellular domain containing five tandemly arranged immunoglobulin-like domains, a transmembrane region, and a cytoplasmic domain (Staunton *et al.*, *Cell 52*:925-933 (1988); Simmons *et al.*, *Nature (Lond.) 331*:624-627 (1988)). ICAM-1 is a ligand for the leukocyte integrins, lymphocyte function antigen-1 (LFA-1; CD11a/CD18) (Rothlein *et al.*, *J. Immunol. 137*:1270-1274 (1986); Marlin *et al.*, *Cell 51*:813-819 (1987)) and Mac-1 (CD11b/CD18) (Diamond *et al.*, *J. Cell. Biol. 111*:3219-3139 (1990); Smith *et al.*, *J. Clin. Invest. 83*:2008-2017 (1989)). The recognition, adhesion, and extravasation of lymphoid and myeloid blood cells through the vascular endothelium is an initial step of host immune response to tissue injury. The CD11/CD18 family of proteins are crucial for leukocyte and myeloid cell adhesion to endothelium, T cell activation, cytotoxic T cell killing, and neutrophil chemotaxis and homotypic aggregation (Larsen *et al.*, *Immunol. Rev. 114*:181 (1990)). ICAM-1 is also subverted as a cellular receptor by the major group of human rhinoviruses (HRV), the etiologic agent of the common cold (Staunton *et al.*, *Cell 56*:849-853 (1989); Greve *et al.*, *Cell 56*:839-847 (1989)). A soluble form of ICAM-1 lacking the transmembrane and cytoplasmic domains binds HRV and inhibits rhinovirus adhesion (Marlin *et al.*, *Nature (Lond.) 344*:70-72 (1990)).

Monoclonal antibody blocking studies have indicated that the binding sites for LFA-1 and HRV are proximal. Analysis of mutant ICAM-1 molecules has demonstrated that mutations in the amino terminal domain have the strongest effect on LFA-1 and HRV binding (Staunton *et al.*, *Cell 61*:243-254 (1990)). Domains D1 and D2

demonstrate a close physical association and appear conformationally linked (Staunton *et al.*, *Cell 52*:925-933 (1988)). Amino acid substitution mutants demonstrate that while the LFA-1 and HRV contact sites overlap, they are distinct (Staunton *et al.*, *Cell 61*:243-254 (1990)). The integrin Mac-1 binds to the third $NH_2$-terminal Ig-like domain of ICAM-1 in contrast to LFA-1 and this binding is influenced by the extent of glycosylation on the ICAM-1 molecule (Diamond *et al.*, *Cell 65*:961-971 (1991)).

The molecular basis for adhesion of malaria-infected erythrocytes to ICAM-1 is not known. Monoclonal antibodies RR1/1 and R6.5 which inhibit binding of LFA-1 and HRV to ICAM-1 have no effect on IRBC binding to purified ICAM-1-coated surfaces (Ockenhouse *et al.*, *J. Infect. Dis. 164*:163-169 (1991)). Recently, we and others have demonstrated that red blood cells infected with mature intracellular forms of the malaria parasite (IRBC) bind to a region located within the amino-terminal immunoglobulin-like domain of ICAM-1 that is distinct from the regions recognized by LFA-1 and rhinovirus (Ockenhouse *et al.*, *Cell 68*:63-69 (1992); and Berendt *et al.*, *Cell 68*:71-81 (1992)).

ICAM-1 has a restricted distribution *in vivo*, and its expression is regulated by LPS and the cytokines TNF, IL-1$\beta$, and interferon-gamma (Dustin *et al.*, *J. Immunol. 137*:245-254 (1986); Pober *et al.*, *J. Immunol. 137*:1893-1896 (1986); Pober *et al.*, *Transplantation 50*:537 (1990)). Bacterial products and/or inflammatory mediators released at sites of local tissue injury induce ICAM-1 mRNA and protein expression in a wide variety of cells. *In vitro*, human umbilical endothelial cells induced with TNF up regulate the surface expression of ICAM-1 and support adhesion of malaria-infected erythrocytes (Berendt *et al.*, *Nature (Lond.) 341*:57-59 (1989)). *In vivo*, individuals with cerebral malaria have higher levels of plasma TNF than individuals with uncomplicated malaria or uninfected

controls. Paradoxically, an inflammatory response initiated in response to malarial infection is used to the parasites' advantage by selectively modulating the expression of receptors to which parasitized erythrocytes attach.

In principle, the receptor binding site on IRBC surfaces should be conserved and selective pressure exerted to maintain minimal structural variation unless compensatory binding to alternate receptors occur. Sequestration of malaria-infected erythrocytes to host endothelium occurs in all persons infected with the parasite regardless of clinical severity. A small percentage of infected individuals, independent of parasitemia, progress to complicated and severe forms of the disease. The precise factors and mechanisms responsible for severe malaria are unknown. While the majority of parasitized erythrocytes from naturally-acquired infections bind only to CD36 *in vitro*, a smaller subpopulation of parasitized erythrocytes from some isolates bind to ICAM-1 and CD36. IRBC bind to different receptors in different tissues depending upon the genetic regulation of host cellular receptors and the parasite cytoadherent phenotype as expressed by single or multiple counter-receptors. Deleterious effects to the host result from the sequestration of a numerically smaller proportion of IRBC expressing the pertinent counter-receptor within a population of parasitized red cells directing the binding of IRBC to capillary endothelium within the brain leading to cerebral malaria.

Antigenically diverse naturally-acquired malaria isolates demonstrate serologically defined infected erythrocyte surface epitopes. Immune sera inhibits IRBC adhesion to human umbilical vein endothelial cells in a strain-specific manner (Udeinya *et al.*, *Nature (Lond.) 303*:429-431 (1983)), and no pan-specific sera has been identified which inhibits IRBC adhesion of geographically diverse malaria isolates.

## SUMMARY OF THE INVENTION

The present invention discloses the binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes. An IRBC binds to the first $NH_2$-terminal domain of human but not mouse ICAM-1. Further, the present invention discloses that small peptides, corresponding to a contiguous sequence of ICAM-1, are capable of inhibiting the binding of an IRBC to ICAM-1. In addition, it is disclosed herein that the binding sites within domain 1 reside spatially distant from the recognition sites for LFA-1 and HRV.

A therapeutic strategy directed toward reversing parasite sequestration ultimately can protect infected individuals from the deleterious complications of vascular occlusion.

Utilizing the present invention, anti-receptor soluble ICAM-1 analogues based upon the critical contact residues for IRBC can now be engineered to bind, lyse, and kill sequestered intraerythrocytic parasites in cases of severe and complicated *falciparum* malaria, as well as diagnosis of the presence of malaria.

The two primary sites an IRBC can bind to on a non-infected cell are ICAM-1 and CD36. Therefore, the binding of an IRBC to an uninfected cell can be inhibited by providing to the cells an agent capable of binding to the ICAM-1 binding site on the IRBC, the IRBC binding site on ICAM-1, the CD36 binding site on the IRBC, or to the IRBC binding site on CD36.

By inhibiting the binding of an IRBC to a non-infected cell, the complications arising from malaria can be ameliorated.

The agents of the present invention include:

(a) agents which are capable of binding to the ICAM-1 binding site on an IRBC, said agents selected from the group consisting of ICAM-1, a fragment of ICAM-1, a functional derivative thereof, a peptide, an

antibody, or a carbohydrate;

(b) agents which are capable of binding to the IRBC binding site on ICAM-1, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate;

(c) agents which are capable of binding to the CD36 binding site on an IRBC, said agents selected from the group consisting of CD36, a fragment of CD36, a functional derivative of CD36, a peptide, an antibody, or a carbohydrate; and

(d) agents which are capable of binding to the IRBC binding site on CD36, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate.

For example, the present invention includes the peptide agent whose amino acid sequence is: GSVLVT (SEQ ID NO 1). This agent is capable of binding to the ICAM-1 binding site of an IRBC.

The invention further includes a method for producing a desired hybridoma cell that produces an antibody which is capable of binding to the IRBC binding site on ICAM-1, the ICAM-1 binding site of an IRBC, the IRBC binding site on CD36, or the CD36 binding site of an IRBC.

The invention further includes chimeric proteins comprising ICAM, or fragments thereof, or CD36, or fragments thereof, fused to an immunoglobulin or a fragment thereof. One such ICAM-1 fusion protein, herein designated F185G1, consists of soluble-ICAM-1 fused to the hinge region and constant domains CH2 and CH3 of human IgG1 heavy chain. One such CD36 fusion protein, herein designated D30F492, consists of soluble-CD36 fused to the hinge region and constant domains CH2 and CH3 of human IgG1 heavy chain.Fusion proteins of this nature have been demonstrated to stimulate phagocytosis of an IRBC when bound to the IRBC's surface.

The invention further includes a method of stimulating phagocytosis of an IRBC in a patient with malaria comprising

administering to said patient a therapeutically effective amount of a fusion protein comprising ICAM-1, or a fragment thereof, and/or CD36, or a fragment thereof, each of which is fused to an immunoglobulin or a fragment thereof.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

Binding of malaria-infected erythrocytes to chimeric forms of ICAM-1. Chimeric molecules were generated as described in Staunton *et al.*, *Cell* 61:243-254 (1990) and transfected into COS cells. The two chimeric molecules are composed as follows: hmICAM-1 (human ICAM-1, domains 1 and 2; murine ICAM-1, domains 3-5) and mhICAM-1 (murine ICAM-1, domains 1 and 2; human ICAM-1, domains 3-5). Results represent the mean of three determinations ± standard deviation.

Figure 2.

Alignment of amino acids in first amino-terminal domain of human ICAM-1, murine ICAM-1, and human ICAM-2. Amino acid substitution mutations within human ICAM-1 affecting binding of *Plasmodium falciparum* IRBC (Pf), LFA-1 (L), and HRV (R) are indicated by the solid line. The alignment of sequences by predicted secondary structure is indicated by β-strands A-G.

Figure 3.

Effect of ICAM-1 peptides on IRBC binding.

A. Inhibition of binding of malaria-infected erythrocytes to ICAM-1 by overlapping synthetic hexapeptides. ItG-ICAM IRBC and ICAM-1 hexapeptides (500 ug/ml) were added to ICAM-1 coated plates for 60 minutes. The peptides were acetylated at the N-terminus, amidated at the C-terminus. Aba is alpha amino butyric acid and is substituted in

sequence for Cys. Results represent the mean ± s.d. of three determinations and are compared to control IRBC binding to ICAM-1 in absence of peptides.

B. Dose-dependent inhibition of IRBC binding to ICAM-1 by peptides $Pro^{12}$-$Thr^{23}$ and GSVLVT and sICAM-1, ItG-ICAM-1 IRBC (closed symbols) and ItG-CD36 IRBC (open symbols) and sICAM-1 or synthetic peptides at concentrations indicated were incubated on plates previously coated with 10 ug/ml ICAM-1 or 1 ug/ml CD36, respectively. Binding of IRBC to adhesion receptors were determined and the results represent the mean per cent binding compared to control samples incubated in PBS alone. Control binding of ItG-ICAM-1 IRBC to purified ICAM-1 is 1578 ± 225 IRBC/$mm^2$ and binding of ItG-CD36 to purified CD36 is 860 ± 108 IRBC/$mm^2$.

Fig. 4.

A. Schematic diagram of the F185G1 expression construct (pCDF185G1) and the F185G1 immunoadhesion.

B. SDS-PAGE of the immunoadhesin. COS cells were transiently transfected with the plasmid pCDF185G1 or as a control CDM8 and labeled with [$^{35}$S] methionine and cysteine. Secreted material was precipitated with protein A-Sepharose and subjected to SDS-PAGE and fluorography. Identical results were obtained with immunoprecipitations with anti-ICAM-1 mAB R6.5 (data not shown).

Fig. 5.

*P. falciparum*-infected erythrocyte and T-lymphoblastoid cell biding to recombinant ICAM-1.

A. Adhesion of ItG-ICAM-1-IRBC to surfaces coated with the indicated concentrations of ICAM-1-IgG1 chimera (F185G1), CHO cell-derived soluble ICAM-1 and *baculovirus*-derived soluble ICAM-1.

B. Inhibition of IRBC adhesion to ICAM-1-coated surfaces by F185G1 chimera, sICAM-1, or human IgG.

C. Binding of T-lymphoblastoid cells + or - PMA to F185G1 coated surfaces.

D. Inhibition of PMA-stimulated SKW-3 adhesion to sICAM-1-coated surface by F185G1.

Fig. 6.

Phagocytosis of *Plasmodium falciparum*-infected erythrocytes by human monocytes.

Fig. 7.

Monocyte phagocytosis of *Plasmodium falciparum*-infected IRBC.

A. CD36-binding IRBC preincubated with F185G1 chimera bind to the monocyte surface but are not phagocytosed.

B and C. ICAM-1-binding IRBC preincubated with F185G1 chimera are phagocytosed and internally degraded by monocytes.

D. ICAM-1-binding IRBC in the absence of ICAM-1 immunoadhesin are not phagocytosed by monocytes.

Conditions for F185G1 mediated IRBC phagocytosis were as described in the Example for Figure 6.

Fig. 8.

Amino acid sequence of CD36. The residue used in generating the chimeras are identified in the sequence.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on the identification of the two primary binding sites an IRBC can bind to on a non-infected cell. These sites are contained on ICAM-1 and CD36. The present invention discloses that the binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes is the first $NH_2$-terminal domain between residues $Gly^{14}$-$Ser^{22}$ of human, but not mouse, ICAM-1. Further, it is disclosed herein that a peptides with an amino acid sequence selected from this region, can block the binding of an IRBC to ICAM-1.

Utilizing the amino acid sequence of the binding site, the present invention provides agents and methods for the treatment and diagnosis of malaria.

### I. Agents of the Present Invention

The present invention includes:

(a) agents which are capable of binding to the ICAM-1 binding site on an IRBC, said agents selected from the group consisting of ICAM-1, a fragment of ICAM-1, a peptide, an antibody, or a carbohydrate;

(b) agents which are capable of binding to the IRBC binding

site on ICAM-1, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate;

(c) agents which are capable of binding to the CD36 binding site on an IRBC, said agents selected from the group consisting of CD36, a fragment of CD36, a peptide, an antibody, or a carbohydrate; and

(d) agents which are capable of binding to the IRBC binding site on CD36, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate.

(e) agents which are capable of stimulating phagocytosis of an IRBC, said agents selected from the group consisting of an immunoglobulin, or fragment thereof, fused to ICAM-1, a fragment thereof, CD36, or a fragment thereof.

These agents are capable of blocking the binding of an IRBC to either ICAM-1 or CD36.

In addition, the present invention includes functional derivatives of the above described agents.

As used herein, a "functional derivative" of an agent of the present invention is an agent which possesses a biological activity that is substantially similar to the biological activity of the agent it is a derivative of. For example, if the agent is capable of binding to the ICAM-1 binding site of an IRBC, then the functional derivative will possess this binding ability. The term "functional derivative" includes "fragments," "variants," and "chimeras" of the parent molecule.

A "fragment" of an agent is meant to refer to any subset of the agent it is derived from. Fragments of ICAM-1 or CD36 which contain IRBC binding activity and are soluble are especially preferred. Soluble fragments of CD36 or ICAM-1 can be rationally designed by one skilled in the art. Generally, soluble fragments are generated by deleting the trans membrane regions of the molecule. Additionally, some of the more hydrophobic regions of the protein can be deleted.

As used herein, a "variant" of a molecule is meant to refer to a molecule substantially similar in structure and function to either the entire molecule, or to a fragment thereof.

A molecule is said to be "substantially similar" to another molecule if both molecules have substantially similar structures or if both molecules possess a similar biological activity. Thus, provided that two molecules possess a similar activity, they are considered variants, as that term is used herein, even if the sequence of amino acid residues is not identical.

As used herein, an agent is said to be a "chimeric-agent" if the agent possesses a structure not found in the agent it is derived from. Such additional structures are added to a parent agent in order to improve one of the agent's physical properties such as solubility, absorption, biological half life, etc., to eliminate or decrease one of the agent's undesirable properties or side effects such as immunogenicity or toxicity, or to add a property to the agent which is not present in the parent agent such as the ability to stimulate a biological effector function such as phagocytosis, complement-dependent cytolysis (CDC), antibody-dependent, cell-mediated cytotoxicity (ADCC), etc. Moieties capable of mediating such effects are disclosed in *Remington's Pharmaceutical Sciences* (1980).

One type of chimeric-agent are "chemical-derivatives." Chemical-derivatives contain one or more additional chemical moieties which are not part of the naturally occurring agent.

"Toxin-derivatized" agents constitute a special class of chemical-derivatives. Toxin-derivatives contain an agent of the present invention covalently attached to a toxin moiety. Procedures for coupling such moieties to a molecule are well known in the art and are generally performed *in situ*.

The binding of a toxin-derivatized agent to a cell brings the toxin moiety into close proximity to the cell and thereby promotes cell death. Any suitable toxin moiety may be employed; however, it is preferable to

employ toxins such as, for example, the ricin toxin, the cholera toxin, the diphtheria toxin, radioisotopic toxins, or membrane-channel-forming toxins.

"Protein-derivatized" agents constitute another type of chimeric-agent. Protein-derivatives contain one or more additional peptide moieties which are not part of the naturally occurring agent. Protein derivatives may be generated *in situ* using chemical means or *in vivo* using recombinant DNA techniques.

"Antibody-derivatized" agents constitute a special class of protein-derivative. Antibody-derivatives contain an agent of the present invention covalently attached to an antibody or antibody fragment. Procedures for coupling such moieties to a molecule are well known in the art.

The binding of an antibody-derivatized agent to a cell brings the antibody or antibody fragment into close proximity to the cell. The antibody fragment will promote cell death by stimulating a biological effector function such as phagocytosis. Any suitable antibody or antibody fragment may be employed depending on the effector function which is to be stimulated (see Bruggeman *et al., J. Exp. Med. 166*:1351-1361 (1987) for a review of effector functions); however, it is preferable to employ a fragment which contains the constant domain of one of the antibody chains such as the hinge and constant regions CH2 and CH3 of the human IgG1 heavy chain.

Antibody derivatives of CD36 are, in general, generated by ligating a DNA sequence encoding a fragment of the entire CD36 molecule into a vector which contains a signal peptide and sequences encoding the desired antibody fragment. The fragments of CD36 which are used in such constructs are preferably deleted for the hydrophobic regions of CD36, residues 6-28 and 439-465. In one aspect of this embodiment residues 1-6 and 466-471 are also deleted. The preferred fragments of CD36 start with an amino acid residue selected from the group consisting of D30, Q34,

Q40, G46, or F50 of CD36 and continue to an amino acid residue selected from the group consisting of N416, F429, V433, G435, or L439.

Functional derivatives of the peptide agents of the present invention having an altered amino acid sequence include insertions, deletion, and substitutions in the amino acid sequence of the agent. These can be prepared by synthesizing a peptide with the desired sequence. While the site for introducing an alteration in the amino acid sequence is predetermined, the alteration *per se* need not be predetermined. For example, to optimize the performance of altering a given sequence, random changes can be conducted at a target amino acid residue or target region to create a large number of derivative which can then be screened for the optimal combination of desired activity.

The effect any particular substitution, deletion, or insertion will have on the biological activity of an agent may be evaluated by routine screening assays by one skilled in the art. For example, a derivative of the IRBC binding site on ICAM-1 is made by synthesizing a polypeptide containing an alteration in the amino acid sequence of ICAM-1. The peptide is then screened for the ability to block IRBC binding to immobilized ICAM-1. Additionally, other screening assays known in the art can be employed to identify a change in a specific characteristic of the agent such as a change in the immunological character, affinity, redox or thermal stability, biological half-life, hydrophobicity, or susceptibility to proteolytic degradation of the functional derivative.

One class of derivatives of the agents of the present invention which are especially preferred are soluble derivatives. Generally, soluble derivatives of a molecule are generated by deleting transmembrane spanning regions or by substituting hydrophilic for hydrophobic amino acid residues.

Another class of derivatives of the agents of the present invention which are based on CD36 which are especially preferred are those agents

which lack the normal CD36 collagen binding site. Such derivatives can be created by generating random mutations via site directed or random mutagenesis and then screening the derivatives for their inability to bind collagen.

As an alternative to random mutagenesis, site directed mutagenesis directed to regions suspected of containing the collagen binding site can be performed. The collagen binding site can be identified by, comparing the amino acid sequence of CD36 with other collagen binding proteins to identify regions of homology, analyzing the amino acid sequence of CD36 for regions which from disulfide bridges, or by cross linking collagen to CD36 and then proteolytically mapping, using agents such as trypsin, the cross-linked protein to identify the collagen linked fragment. Once the collagen binding region is identified, linker scanning mutagenesis can be employed to optimize the directed nature of the mutagenesis.

The agents of the present invention may be obtained by: natural processes (for example, by inducing an animal, plant, fungi, bacteria, etc., to produce a peptide corresponding to a particular sequence, or by inducing an animal to produce polyclonal antibodies capable of binding to a specific amino acid sequence); synthetic methods (for example by synthesizing a peptide corresponding to the IRBC binding site on ICAM-1, or a functional derivatives of said peptide); by hybridoma technology (for example, by producing monoclonal antibodies capable of binding to the IRBC binding site on ICAM-1); or recombinant technology (such as, for example, to produce the agents of the present invention in diverse hosts (i.e., yeast, bacteria, fungi, cultured mammalian cells, etc.)), using a recombinant plasmid or viral vectors). The choice of which method to employ will depend upon factors such as convenience, desired yield, etc. However, it is not necessary to employ only one of the above-described methods, processes, or technologies to produce a particular anti-inflammatory agent; the above-described processes, methods, and

technologies may be combined in order to obtain a particular agent.

## A. Antibodies

The antibodies of the present invention can be generated by a variety of techniques known in the art.

The antibodies of the present invention include monoclonal and polyclonal antibodies, as well fragments and humanized forms of these antibodies. Humanized forms of the antibodies of the present invention may be generated using one of the procedures known in the art such as chimerization or CDR grafting.

In general, techniques for preparing both polyclonal and monoclonal antibodies are described elsewhere (Campbell, A.M., "*Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*," Elsevier Science Publishers, Amsterdam, The Netherlands (1984)).

The invention provides an antibody, and especially a monoclonal antibody, capable of binding to a molecule selected from the group consisting of the IRBC binding site on ICAM-1, the ICAM-1 binding site on an IRBC, the IRBC binding site on CD36, and the CD36 binding site on an IRBC.

An antibody which binds to the IRBC binding site on ICAM-1 can be generated using a synthetic polypeptide whose amino acid sequence is identical to the amino acid sequence of the IRBC binding site on ICAM-1 as an antigen for immunizing an animal. One such peptide for generating an antibody which binds to the IRBC binding site on ICAM-1 has the following amino acid sequence: GSVLVT (SEQ ID NO 1).

An antibody which binds to the ICAM-1 binding site on an IRBC can be generated by immunizing an animal with an IRBC. The antisera is then screened for its ability to block an IRBC from binding to

immobilized ICAM-1.

An antibody which binds to the CD36 binding site on an IRBC can be generated by immunizing an animal with an IRBC. The antisera is then screened for its ability to block an IRBC from binding to immobilized CD36.

An antibody which binds to the IRBC binding site on CD36 can be generated by immunizing an animal with CD36. The antisera is then screened for its ability to block an IRBC from binding to immobilized CD36.

One skilled in the art will be able to readily obtain both polyclonal and monoclonal antibodies with the above described specificities using procedures known in the art (Lutz *et al., Exp. Cell Res. 175*:109-124 (1988), Campbell, A.M., *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984).

The polypeptide may be modified or administered in an adjuvant in order to increase the peptide antigenicity. Methods of increasing the antigenicity of a polypeptide are well known in the art. Such procedures include coupling the antigen with a heterologous protein (such as globulin or $\beta$-galactosidase) or through the inclusion of an adjuvant during immunization.

B. Peptides

The peptides of the present invention can be generated by a variety of techniques known in the art. The peptides of the present invention include peptides whose amino acid sequence is substantially homologous to the naturally occurring binding sites disclosed herein as well as peptides generated through rational design which possess a desired binding specificity but differ significantly in amino acid sequence from the naturally occurring binding site.

As used herein a peptide is said to have an amino acid sequence substantially homologous to another if, due to the presence of common amino acid residence in homologous positions, the two peptides share common biological of physical property.

In general, techniques for preparing synthetic peptides with a defined sequence or structure are well known in the art.

The peptides of the present invention whose amino acid sequences are substantially homologous to the naturally occurring binding site include; the ICAM-1 binding site of an IRBC, the CD36 binding site of an IRBC, the IRBC binding site on ICAM-1, and the IRBC binding site on ICAM-1.

One such peptide, SEQ ID NO 1, has an amino acid sequence which is homologous to the IRBC binding site on ICAM-1.

In addition to peptides whose sequence, are substantially homologous to the naturally occurring binding site, one skilled in the art can readily generate, through rational design, peptides that possesses the ability to bind to a specific amino acid sequence or antigenic epitope (Hodgson, J, *Biotechnology 8:*1245-1247 (1990)). Computer modeling systems are available that allow one skilled in the art to design a peptide which is able to bind to the specific regions and sequences disclosed herein. The peptide which are made according to this method can be readily screened for a desired specificity and physical properties.

C. Carbohydrates

In addition to proteins, carbohydrates can be rationally designed to block protein/protein binding (Hodgson,J. Biotechnology *9*:609-613 (1991)).

Based on the present disclosure a carbohydrate can now be designed to block an IRBC from binding to ICAM-1 or to block an IRBC

from binding to CD36.

II. Therapeutic Uses of the Agents of the Present Invention

Specifically, the invention includes the use of the agents disclosed herein; a) to inhibit the binding of an IRBC to a non-infected cell, and b) to preferentially kill an IRBC.

In detail, the binding of an IRBC to ICAM-1 can be inhibited by providing an effective amount of an agent capable of binding to either the IRBC binding site on ICAM-1 or the ICAM-1 binding site on a IRBC. The binding of an IRBC to CD36 can be inhibited by providing an effective amount of an agent capable of binding to either the IRBC binding site on CD36 or the CD36 binding site on a IRBC. An example of an agent capable of inhibiting the binding of an IRBC to ICAM-1 is a peptide whose sequence is shown in SEQ ID NO 1. By providing such an agent to a mammal, some of the deleterious effects of malaria can be ameliorated.

An IRBC can be preferentially killed by providing an IRBC with a toxin derivatized agent which is capable of selectively binding the IRBC. Examples of such agents include a peptide of SEQ ID NO 1 or an antibody which is capable of binding to either the ICAM-1 or the CD36 binding site on an IRBC covalently liked to a toxin such as ricin. By providing such a molecule to a mammal, the IRBC can be preferentially killed.

Alternatively, an IRBC can be preferentially killed by utilizing a mammal's natural defense systems. Specifically, by providing an IRBC with an antibody-derivatized agent which is capable of selectively binding the IRBC, the constant regions of the antibody moiety of the antibody-derivative agent will stimulate biological activities such as phagocytosis, CDC, and ADCC. Examples of such agents include F185G1 and D30F429

which consist of the hinge region and constant domains CH2 and CH3 of the human IgG1 heavy chain covalently linked to a soluble derivative of ICAM-1 or CD36 respectively. By providing such a molecule to a mammal, phagocytosis of an IRBC can be stimulated.

III. Administration of the Agents of the Present Invention

The agents of the present invention may be administered to a mammal singly or in combination with each other. Most preferably, an agent based on ICAM-1 is administered in combination with an agent based on CD36.

The agents of the present invention may be administered intravenously, intramuscularly, subcutaneously, enterally, topically or other non-enteral means. When administering antibodies or peptides by injection, the administration may be by continuous injections, or by single or multiple injections.

The agents of the present invention are intended to be provided to recipient mammal in a "pharmaceutically acceptable form" in an amount sufficient to "therapeutically effective."

An amount is said to be therapeutically effective if the dosage, route of administration, etc. of the agent are sufficient to block the binding of an IRBC with a defined molecule or is sufficient to kill a portion of the IRBCs present in the mammal. For example, an agent of the present invention when provided to a mammal to block the binding of an IRBC to ICAM-1 is said to be therapeutically effective if it is provided in sufficient dosage to block IRBC/ICAM-1 binding.

The administration of the agents of the present invention may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the agent is provided in advance of any malaria symptomology. The prophylactic administration of the agent serves to

prevent or attenuate any subsequent spread of the malaria parasite. When provided therapeutically, the agent is provided at (or shortly after) the onset of a symptoms of the actual infection. The therapeutic administration of the compound(s) serves to attenuate or ameliorate any actual symptoms.

An agent is said to be "pharmacologically acceptable form" if its administration can be tolerated by a recipient patient. The agents of the present invention can be formulated according to known methods of preparing pharmaceutically useful compositions, whereby these materials, or their functional derivatives, are combined with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation, inclusive of other human proteins, e.g., human serum albumin, are described, for example, in Remington's *Pharmaceutical Sciences* (16th ed., Osol, A., Ed., Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition which is suitable for effective administration, such compositions will contain an effective amount of an agent of the present invention together with a suitable amount of carrier. In addition to carriers, the antibodies of the present invention may be supplied in humanized form, through chimerization or CDR grafting, when administered to a human in order that the antibody is in a more "pharmacologically acceptable form."

Additional pharmaceutical methods may be employed to control the duration of action of the agents of the present invention. Control release preparations may be achieved through the use of polymers to complex or absorb the agents of the present invention. The rate and duration of the controlled delivery may be regulated to a certain extent by selecting an appropriate macromolecule matrix, by varying the concentration of macromolecules incorporated, as well as the methods of incorporation. Another possible method to control the duration of action by controlled release preparations is to incorporate the agents of the

present invention into particles of a polymeric material, such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinyl acetate copolymers. Alternatively, instead of incorporating these agents into polymeric particles, it is possible to entrap these materials in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, by gelatine or poly(methylmethacylate) microcapsulation, or in colloidal drug delivery systems, for example, liposomes, albumin microspheres, microemulsions, nanoparticles, and nanocapsules or in macroemulsions.

## IV. Diagnostic Use of the Agents of the Present Invention

The agents of the present invention can be used to; a) diagnose the presence of an IRBC in a mammal, and b) determine the location of the IRBC in a mammal.

### A. Modifications of the Agents of the Present Invention

One skilled in the art can: a) detectably label the agents of the present invention using radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horse radish peroxidase, alkaline phosphatase, etc.) fluorescent labels (such as FITC or rhodamine, etc.), or paramagnetic atoms, using procedures well-known in the art, for example see Sternberger, L.A. *et al., J. Histochem. Cytochem. 18*:315 (1970), Bayer, E.A. *et al., Meth. Enzym. 62*:308 (1979), Engval, E. *et al., Immunol. 109*:129 (1972), Goding, J.W. *J. Immunol. Meth. 13*:215 (1976); or b) immobilized the agents of the present invention on a solid support of; plastic such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads (Weir, D.M. *et al.*, "*Handbook of Experimental Immunology*" 4th Ed.,

Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986), Jacoby, W.D. *et al., Meth. Enzym. 34* Academic Press, N.Y. (1974)).

1. Detectably Labeled Agents

In detectably labeled form, the agents of the present invention can be used to: a) assay for the presence of an IRBC *in vivo* as well as *in vitro*; and b) localize the presence of an IRBC to a specific location *in vivo*. One skilled in the art can readily incorporate the labeled agents of the present invention into any of the currently available *in vivo* or *in vitro* assay formats such as an ELISA assay, a latex agglutination assay, and magnetic resonance imaging.

2. Immobilized Agents

In immobilized form, the agents of the present invention can be used to: a) purify an IRBC from a population containing non-infected cells; and b) be used in the assay formats described above.

An IRBC can be purified from a population of cells using affinity chromatography. Specifically, an infected cell expressing either the ICAM-1 or CD36 binding site can be isolated from a mixture of cells by passing the cells over a column which contains an immobilized agent capable of binding the ICAM-1 or CD36 binding site present on the infected cell.

Having now generally described the invention, the agents and methods of obtaining same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention, unless specified.

## EXAMPLES

The attachment of erythrocytes infected with the parasite, *Plasmodium falciparum*, to human capillary and post-capillary venular endothelium is the primary step leading to complications, from severe and cerebral malaria. The intercellular adhesion molecule-1 (ICAM-1, CD54) has been implicated as a cytoadhesion receptor for *Plasmodium falciparum*-infected erythrocytes. Wild type and mutant ICAM-1 expressed in COS cells were examined for binding to laboratory-adapted and naturally-acquired malaria-infected erythrocytes. Domain deletion, human-mouse chimeric ICAM-1 molecules, and amino acid substitution mutants localized the primary binding site for parasitized erythrocytes to the first $NH_2$-terminal immunoglobulin-like domain of ICAM-1. The ICAM-1 binding sites are distinct from those recognized by LFA-1, Mac-1, and the human major-type rhinoviruses. The addition of overlapping synthetic peptides encompassing the binding site on ICAM-1 inhibited malaria-infected erythrocyte adhesion to recombinant soluble ICAM-1-coated surfaces. These findings form the basis of and facilitate in the construction of soluble ICAM-1 or soluble CD36 derivatives targeted at preventing and reversing the malaria-infected sequestration to host endothelium in the peripheral circulation vascular bed.

## EXPERIMENTAL PROCEDURES

### Generation of ICAM-1 Mutants

Oligonucleotide-directed mutagenesis (Kunkel, T.A., *Proc. Natl. Acad. Sci USA 82*:488-492 (1985)) was used to generate ICAM-1 deletion, chimeric, and amino acid substitution mutants as described (Staunton *et al., Cell 61*:243-254 (1990)).

Transfection of COS Cells

COS cells at 50% confluency were transfected by the DEAE-dextran method using vector alone or vector containing wild-type or mutant ICAM-1 cDNA. COS cells were harvested 72 hours after transfection and the efficiency of transfection of ICAM-1 constructs was analyzed by indirect immunofluorescence and flow cytometry using anti-human ICAM-1 MAbs CL203 (Maio *et al.*, *J. Immunol. 143*:181-185 (1989)) (a gift of Dr. S. Ferrone), and RR1/1 (Dustin *et al.*, *J. Immunol. 137*:245-254 (1986)); and anti-murine MAb YN1/1 (Takei, F., *J. Immunol. 134*:1403-1407 (1986)) (a gift of Dr. F. Takei, Vancouver, B.C.) as previously described (Staunton *et al.*, *Cell 61*:243-254 (1990)).

Parasites

A *Plasmodium falciparum* cloned parental line, ItG-2F6, was selected for increased adhesion to purified ICAM-1 (ItG-ICAM) or to purified CD36 (ItG-CD36) by panning the parasitized erythrocytes on ICAM-1-coated or CD36-coated surfaces (Ockenhouse *et al.*, *Proc. Natl. Acad. Sci. USA 88*:3175-3179 (1991)). Parasites were maintained in continuous culture, synchronized, and enriched for mature trophozoite and schizont stages (35-50% parasitemia) by gelatin flotation. Two naturally-acquired isolates obtained from Thai patients with uncomplicated malaria (CY25), or severe cerebral malaria (Gl5) were adapted to continuous culture and used within 10 cycles of multiplication.

Peptides

ICAM-1 peptides $Pro^{12}$-$Thr^{23}$ and overlapping hexapeptides spanning residues $Gln^{1}$-$Thr^{23}$ were synthesized on an Applied Biosystems peptide synthesizer.

IRBC Binding Assay

Transfected COS cells in RPMI 1640 plus 10% fetal bovine serum were reseeded (2.5 - $4x10^4$/well) 24-48 hours prior to assay into 24-well tissue culture plates at 37°C in 5% $CO_2$. Malaria-infected erythrocytes (400 ul/well; 2% hematocrit; 20-35% parasitemia) were added to COS cells and incubated for one hour at 37°C with occasional rocking. Unattached erythrocytes were removed by rinsing the wells with RPMI 1640. To identify those cells expressing wild-type or mutant ICAM-1 from untransfected cells, the anti-ICAM-1 MAbs CL203 or RR1/1 (5 ug/ml) were added to each well. After 45 minutes incubation at room temperature, the wells were washed twice with RPMI 1640, and the cells were fixed with an ice-cold acetone-methanol (50% v/v) mixture for one minute. Cells were rinsed with PBS and colloidal gold-labelled anti-mouse antibody (Amersham, Arlington, IL) was added to each well for 30 minutes, followed by three washes with phosphate-buffered saline. A silver enhancement reagent (IntenSEM, Amersham, Arlington Heights, IL) which amplifies the colloidal gold signal was added and the reaction was terminated after 20 minutes. Cell-bound IRBC and surface ICAM-1 were easily identified under phase contrast microscopy. Cells were fixed with 2% glutaraldehyde, stained with Giemsa, and bound IRBC were quantitated under light microscopy by an unbiased observer. Binding of IRBC to ICAM-1 mutants was expressed as a percentage of IRBC adhesion to wild-type ICAM-1 transfected cells.

IRBC binding to ICAM-1-coated or CD36-coated surfaces was performed as follows. Soluble ICAM-1 (10ug/ml) (Marlin *et al., Nature (Lond.) 344*:70-72 (1990)) or CD36 (1 ug/ml) (Tandon *et al., J. Biol. Chem. 264*:7576-7583 (1989)) was coated onto plastic petri dishes (10 ug/ml) overnight at 4°C. PBS containing BSA (1%) was added for 60 minutes to block non-specific binding. Malaria-infected erythrocytes (final concentration 0.5%), ItG-ICAM-1 or ItG-CD36, which bind to ICAM-1 or CD36, respectively, were added to the receptor-coated plates for 1 hour, rinsed carefully to remove unattached erythrocytes, fixed with 2% glutaraldehyde/PBS and stained with Giemsa stain. In some experiments ICAM-1 peptides were preincubated for 30 minutes with the IRBC prior to addition to receptor-coated plates. The number of IRBC bound per $mm^2$ surface area was quantitated by light microscopy.

LFA-1 and HRV Binding Assays

The binding of ICAM-1 mutants to petri dishes coated with immunoaffinity-purified LFA-1 was performed as previously described (Diamond *et al., J. Cell. Biol. 111*:3219-3139 (1990)). Human rhinovirus major type 14 binding to COS cells transfected with mutant ICAM-1 was performed as described (Staunton *et al., Cell 61*:243-254 (1990) herein incorporated by reference).

Construction of the F185G1 Immunoadhesin

A 1.3kb fragment containing the γ1 hinge, $C_H2$ and $C_H3$ sequence was generated by PCR from a plasmid containing the human gene (Traunecker *et al., Nature 339*:68-70 (1989)) using oligonucleotide primers 5'TTTCTCGAGGGTGTCTGCTGGAAGCAGGCTCAG (Seq. ID No. 10) and 5'TTTGCGGCCGCTGGGAGCGGGGCTTGCCGGCCGTCG

(Seq. ID No. 11). The 5′ Xho1 and 3′ Not1 sites introduced by the primers were used to subclone the IgG1 sequence into pCDM8 to produce pCDG1. To construct an ICAM-1-IgG1 chimera, a PCR fragment was generated that contains the ICAM-1 cDNA sequence for signal peptide and domains 1 and 2 terminates with the codon F185 (Staunton *et al., Nature 339*:61-64 (1989))using primers 5′ACCGGAAGCTTCTAGAGATCCCTCGACCACGAGATCCATTG TGC (Seq. ID No. 12) and 5′TTCTGAGTCTCACCAAAGGTCTGGAGCTGGTAGGGGGC (Seq. ID No. 13). The fragment contains a 5′ HindIII site, a translational stop codon following the codon for F185, the 5′ donor splice that follows the γ1 $C_H$1 exon, and a 3′ Xhol site. This fragment was subcloned into HindIII and Xho1 sites of pCDG1 to produce pCDG185G1. Culture supernatants of COS cells transfected with pCDG185G1 contained approximately 0.5 μg/ml ICAM-1-IgG1 chimera (F185G1) as determined by ELISA on day 3 post transfection. F185G1 was purified from culture media of transfected COS cells by ICAM-1 mAB (R6.5)-Sepharose and protein A-Sepharose chromatography. Figures 4a and b.

Construction of CD36 Immunoadhesin

We wished to couple the extracellular domain of CD36 with the hinge and constant regions of immunoglobulin heavy chains to make a chimeric molecule that would bind to malaria-infected erythrocytes, inhibiting sequestration in the microcirculation, and opsonize infected erythrocytes for phagocytosis by white blood cells. Tandon *et al. J. Biol. Chem. 264*:7570 (1989) described the N-terminal sequence of CD36 and its content of a hydrophobic region. Oquendo *et al., Cell 58*:95 (1989) subsequently described the complete cDNA sequence. Oquendo *et al.* reviewed Tandon *et al.*'s information that the mature sequence began with

the second translated amino acid and contained a hydrophobic stretch near the N-terminus, but stated that "It is not clear whether the single *Arg* residue preceding the hydrophobic region would be sufficient to allow amino-terminal membrane anchoring." Based on the prior art of Tandon *et al.* and their own findings, Oquendo *et al.* believed that this would not be a stop transfer sequence because they include the N-terminal hydrophobic region as part of the extracellular domain. This is clear from their discussion and Figure 4, which does not show an N-terminal transmembrane region, but only a single C-terminal transmembrane region at amino acids 439-465 (numbering refers to the mature amino acid sequence, lacking the N-terminal methionine). CD36 shows no homology to other proteins, and therefore there are no precedents that suggest domains that could be used for the design of chimeras.

We initially constructed chimeras containing amino acid residues 1-439 of CD36 fused to the hinge, CH2, and CH3 domains of human $\gamma 1$ heavy chain. However, this construct was unexpectedly not expressed.

We therefore hypothesized that the N-terminus of CD36 might be membrane bound, rather than folded up with the extracellular domain, and made constructs deleted in the N-terminal as well as C-terminal hydrophobic regions. It was impossible to predict the best place to truncate the CD36 molecule relative to the hydrophobic regions, because often "connector" segments are present between the globular extracellular domain and membrane spanning segments. In the preferred embodiment CD36 is deleted for hydrophobic regions, residue 6-28, and 439-465. In one aspect of this embodiment residues 1-5 and 466-471 are also deleted. In the most preferred embodiment, constructs were made so that a signal sequence was spliced to amino acids D30, Q34, Q40, G46, or F50 of CD36. The CD36 sequence continued to amino acid N416, F429, V433, G435, or L439 of CD36, and was then spliced to the hinge, CH2, and CH3 exons of human IgG1 heavy chain. Constructs were transfected into

COS cells. Expression was measured by ELISA using protein A as capture reagent and CD36 MAb as detector reagent or with protein A Sepharose. Thus far, good expression has been obtained with chimeric constructs including the following amino acid segments of CD36: D30F429, Q34L439, and D30N416. This suggests that preferred embodiments contain a portion of CD36 beginning with amino acids 30-34, and ending with amino acids 416 to 439.

In general, the CD36-IgG1 chimeras were constructed by ligating CD36 PCR fragments lacking the transmembrane domain sequences to the expression vectors CDIG1 and CDBG1. CDIG1 and CDBG1 were derived by inserting an Ig and a $\beta$2 microglobin signal peptide sequence and stuffer sequence into the HindIII/XhoI sites of CDG1 respectively. The signal peptide sequence and the 5' UT sequence of ICAM-1 were generated by PCR using a long antisence primer containing the signal peptide sequence and 24bp of ICAM-1 5'UT.

To prepare vectors for ligation of the CD36 fragments, the vectors were digested with ECOR47III(CDIG1) os KASI(CDBG1) and XhoI and then purified via electrophoresis in a low melt agarose gel. The KasI site was blunt ended with Klenow prior to XhoI digestion. The blunt ended vectors terminate with the codon for the -1 position of the signal peptides.

CD36 PCR fragments with a 5' blunt end terminating with the amino terminal codons, and a XhoI site and donor splice sequence were ligated to CDIG1 and CDBG1. The resulting chimeras were expressed in COS cells as described earlier.

The culture supernatants were assayed in an ELISA assay for reactivity to a CD36 mAb and an anti-human IgG Horseradish peroxidase (HRP) conjugate.

IRBC Binding to Immobilized s-ICAM-1

Soluble ICAM-1 truncated before the hydrophobic transmembrane region was purified from the supernatants of transfected CHO cells (Marlin *et al., Nature 344*:70-72 (1990)) or *baculovirus*-vector infected insect cells (Diamond *et al., Cell 65*:961-971 (1991)). For IRBC binding ICAM-1 was adsorbed (20 µl aliquots) to plastic bacteriological plates (Falcon 1007) overnight at 4°C. F185G1 (ICAM-1-IgG1 chimera) was similarly absorbed to plastic plates which had previously been coated with protein A (50 µg/ml). Unbound sites were blocked for 30 minutes at room temperature with 1% BSA-PBS to reduce non-specific binding. Laboratory-adapted intraerythrocytic *P. falciparum* parasites selected *in vitro* to bind to purified ICAM-1 (ItG-ICAM) (Ockenhouse *et al., J. Infec. Dis. 164*:163-169 (1991)) were maintained in synchronous continuous culture and used in adhesion assays at the trophozoite/schizont stage of development. The IRBC were added to ICAM-1-coated plates (40-50% parasitemia, 1% hematocrit) for one hour at room temperature. In inhibition assays, ItG-ICAM IRBC were incubated in solution with increasing concentrations of F185G1 chimera, sICAM-1/CHO, or normal human IgG for 30 minutes prior to addition to plates coated with sICAM-1/CHO (10 µg/ml). Erythrocytes not attached to the sICAM-1-coated surface were removed by gentle rinsing of the plates. Cells were fixed with 2% glutaraldehyde and stained with Giemsa. The number of malaria-infected erythrocytes bound per $mm^2$ surface are represents the mean of three separate determinations. The concentrations of sICAM-1 and F185G1 was determined with a capture ELISA assay (Marlin *et al., Nature 344*:70-72 (1990)), using sICAM-1/CHO as a standard, Figure 5.

For SKW-3 cell binding, F185G1 at the concentration indicated

was absorbed to 96-well microtiter plates which had previously been coated with protein A (20 μg/ml) and blocked with 1% BSA-PBS. SKW-3 cells in binding buffer (RPMI/10% FBS/20mM HEPES) were treated with or without 100 ng/ml PMA for 15 minutes at 37°C and then labeled with 2',7'-bis(2-carboxyethyl)-(5 and 6)-carboxyfluorecein acetomethyl ester (Molecular Probes, Eugene, Or.). Binding ($10^5$ cells/well) was for 1 hour at 25°C.

For F18561 inhibition of SKW-3 binding 96-well microtiter plates were coated with 50 μl sICAM-1 (10 μg/ml, 2 hours, 37°C) and blocked with 1% BSA-PBS. PMA treated SKW-3 (10-cells) were incubated for 30 minutes in 50 μl of binding buffer, with or without F18561 or mAb TS1/18 to the LFA-1 β subunit (1:100 ascites) and then added directly to sICAM-1 coated wells. Binding was for 1 hour at 37°C. Unbound cells were removed by inverting microtiter plates in a tank of PBS/1mm Mg++/.5mM Ca++/0. 1% BSA for 45 minutes. Bound cells were quantitated on a fluorescence concentration analyzer (Pandex). Percent bound (±SD) was calculated by subtracting background binding to wells that were not coated with ICAM-1 from binding to ICAM-1 coated wells, divided by input fluorescence x 100.

Assay for the Phagocytosis of an IRBC

Human mononuclear cells isolated from whole blood by centrifugation on a Ficoll-Hypaque density gradient were washed three times in RPMI 1640 and resuspended in medium supplemented with 10% normal human serum. Cells ($10^5$ in 100 μl) were added to glass coverslips for 90 minutes at 37°C in 7.5% $C0_2$. Non-adherent cells were removed by washing coverslips three times. Attached cells were 95% monocytes by Wright-Giemsa and est rase stains. IRBC (5 X $10^6$ per 100 μl) selected *in vitro* for binding to ICAM-1 (ItG-ICAM) or CD36 (ItG-CD36) were

incubated with F185G1 chimera or normal human IgG (20 μg/ml final concentration) for 30 minutes prior to addition to monolayers of adherent freshly isolated human monocytes. After two hours incubation at 37°C, unattached red blood cells were removed by washing coverslips three times with RPMI 1640. In order to avoid quantitating IRBC attached to the phagocyte surface but not internalized, coverslips were rinsed in hypotonic 0.85% $NH_4Cl$ to lyse attached IRBC. Preincubation of monocyte monolayers with anti-CD36 monoclonal antibody OKM5 completely blocked adhesion of ItG-CD36 infected to monocytes without any effect on subsequent phagocytosis of ItG-ICAM malaria-infected erythrocytes (not shown). Coverslips were fixed with 2% glutaraldehyde followed by staining with Giemsa. The percentage of monocytes which contained intracellular intact infected red cells or degraded parasite pigment was quantitated by light microscopy. Results indicate the mean ±SD of three determinations, Figure 6.

## Example 1

### IRBC Binding To ICAM-1 Deletions

Mutant cDNA clones representing deleted domains $D3^-$ (residues F185-P284), $D4^-$ (P284-L366), and $D4^-D5^-$ (P284-S449) were expressed in COS cells and assayed for IRBC adhesion. Laboratory-adapted infected erythrocytes (ItG-ICAM) selected *in vitro* by repeated panning on ICAM-1-coated surfaces bound to COS cells expressing wild-type ICAM-1 but not to mock-transfected cells nor to cells transfected with ICAM-2 (Table 1). IRBC adhesion to cells was retained after deletion of domains D3-D5 (Table 1). The somewhat decreased adhesion (2-fold) of IRBC to cells transfected with $D3^-$, $D4^-$, or $D4^-D5^-$ can be explained in part to decreased expressions of ICAM-1, as determined by cytofluorimetry, and

to decreased accessibility of binding sites due to the shortening of the ICAM-1 molecules. Binding was specific, since IRBC selected *in vitro* to bind to human CD36 did not bind wild-type nor mutant ICAM-1. IRBC from individuals with uncomplicated malaria, CY25, or complicated severe cerebral malaria, GI5, were cultured *in vitro* for 24 hours to allow intraerythrocytic parasite maturation to the trophozoite stage of development. These infected erythrocytes bound to COS cells expressing wild-type and domain deleted ICAM-1 (Table 1).

## Example 2

### IRBC Binding To Human-Mouse Chimeric ICAM-1

To confirm that domains 1 and 2 of ICAM-1 mediate IRBC adhesion, human-mouse chimeric ICAM-1 molecules were assayed for IRBC binding. The human (Staunton *et al.*, *Cell 52*:925-933 (1988); Simmons *et al.*, *Nature (Lond.) 331*:624-627 (1988)) and murine ICAM-1 (Horley *et al.*, *EMBO J. 8*:2889 (1989)) amino acid primary sequence is 50% identical and each molecule contains 5 Ig-like domains enabling amino terminal chimeric exchanges. Human and murine mutant chimeric ICAM-1 molecules were constructed from cDNAs containing a conserved Bg1 II restriction site at amino acid residue 168 of the human sequence (Staunton *et al.*, *Cell 61*:243-254 (1990). Human domains D1 and D2 (hmICAM-1) or murine domains D1 and D2 (mhICAM-1) were recombined with domains D3-D5 of the other species. The chimeric cDNAs were expressed in COS cells and IRBC binding determined. The efficiency of expression was determined using two MAbs to human ICAM-1, RR/1 and CL203, and MAb YN1/1 (Horley *et al.*, *EMBO J. 8*:2889 (1989)) which recognizes an epitope confined to D1 and D2 of

murine ICAM-1. COS cells which express human but not murine wild-type ICAM-1 bind IRBC (Fig. 1). Furthermore, IRBC bind to hmICAM-1 but not mhICAM-1 (Fig. 1), thus the first 168 residues of human ICAM-1 are sufficient to support binding of an IRBC counter-receptor.

## Example 3

### IRBC Binding To ICAM-1 Substitution Mutants

Amino acid substitution mutants of ICAM-1 have profound effects on LFA-1, Mac-1, and human rhinovirus binding. Similarly, the adhesion of IRBC to single and multiple amino acid substitution mutants was examined. Amino acid substitutions in D1 and D2 are denoted by one-letter code for the wild-type sequence followed by a slash and the one letter code for the mutant sequence (Table 2). The efficiency of mutant ICAM-1 expression on COS cells was determined using MAb CL203 by immunocytofluorimetry and in adhesion assays by immunogold silver staining. Mab CL203 which recognizes an epitope located within the D4 region had no effect on IRBC binding.

The amino acid substitution mutants, D60S/KL and R13G/EA, which conformationally disrupt the secondary structure of domains 1 and 2 (Staunton *et al.*, *Cell 61*:243-254 (1990)) also abrogate IRBC adhesion (Table 2). A two amino acid substitution mutant G15S/SA abrogated IRBC adhesion (Table 2) but had no effect on LFA-1 binding, HRV binding, or binding of MAbs to three different epitopes in D1 and D2 indicating that the overall conformation of the mutant ICAM-1 molecule was preserved. $Gly^{15}$-$Ser^{16}$ residues are highly conserved in human (Staunton *et al.*, *Cell 52*:925-933 (1988); Simmons *et al.*, *Nature (Lond.) 331*:624-627 (1988)) and murine ICAM-1 (Horley *et al.*, *EMBO J. 8*:2889 (1989)) and human ICAM-2 (Staunton *et al.*, *Nature (Lond.) 339*:61-64

(1989)) (Fig. 2). Hence to further characterize the binding site, five additional single amino acid substitution mutants were generated based upon primary structural differences between the human and murine ICAM-1 sequences (Fig. 2). Substitution of $Leu^{18}$ (hmICAM-1) for $Gln^{18}$ (mhICAM-1) resulted in marked loss of IRBC binding to transfected COS cells (Table 2). In contrast, 37 other mutations in domain 1 and 13 mutations in domain 2 including the two potential N-linked glycosylation sites had no effect on IRBC adhesion.

The predicted secondary structure of ICAM-1 based on X-ray crystallographic studies of the immunoglobulin-like molecules (Williams *et al.*, *Annu. Rev. Immunol.* *6*:381-405 (1988); Hunkapiller *et al.*, *Adv. Immunol.* *44*:1-63 (1989)) and on primary amino acid sequences indicate that each Ig-like domain is composed of 7 expected anti-parallel $\beta$-strands folded into a sandwich comprising two facing $\beta$-sheets connected by intramolecular disulfide bonds between strands B and F (Fig. 2). $\beta$-strands A, B, E, D form one sheet while C, F, G strands fashion the opposing sheet. The contact site for *Plasmodium falciparum*-infected erythrocytes is predicted to be localized in domain 1 to a loop between $\beta$ strands A and B and extend into $\beta$ strand B. This contact site is distinct from the binding sites for LFA-1 and HRV (Fig. 2).

## Example 4

### Blocking IRBC Binding To ICAM-1 With Synthetic Peptides

There is another important contrast between the current findings for the *Plasmodium falciparum* sequestration binding site and the previous findings for LFA-1 and rhinovirus. The malaria-infected erythrocyte binding site is highly localized within the sequence, whereas the sites affecting LFA-1 and rhinovirus are noncontiguous within the sequence

suggesting that different segments of the polypeptide chain are folded together to form the contact surface (active site). To determine if ICAM-1 analogues based upon the IRBC binding site within domain 1 would affect IRBC binding to ICAM-1, a synthetic peptide spanning amino acids $Pro^{12}$-$Thr^{23}$ and overlapping hexapeptides were assayed for inhibition of IRBC binding to ICAM-1-coated or CD36-coated surfaces. The inhibitory effect of these peptides was compared to the effect that recombinant soluble ICAM-1 (domains 1-5) (Marlin *et al.*, *Nature (Lond.) 344*:70-72 (1990)) has on IRBC binding to immobilized ICAM-1-coated surfaces. Hexapeptides spanning $Gly^{14}$-$Ser^{22}$ effectively inhibited the binding of ItG-ICAM-infected erythrocytes to ICAM-1-coated plates, while overlapping peptides flanking these regions did not inhibit binding (Fig. 3a). A linear peptide $Pro^{12}$-$Thr^{23}$ and the hexapeptide GSVLVT inhibited IRBC binding in a dose-dependent manner with 50% inhibition at approximately 0.125 and 0.3mM, respectively (Fig. 3b). The inhibitory effect of these peptides was three orders of magnitude less than that observed using sICAM-1 as the inhibitor of IRBC binding (Fig. 3b). The inhibition by the ICAM-1 peptides was specific for ICAM-1-binding infected erythrocytes, since parasitized red cells which bind to an alternative sequestration receptor, CD36, were not inhibited from binding to immobilized CD36 (Fig. 3b). These results confirm that the counter-receptor on the malaria-infected erythrocyte surface for ICAM-1 is functionally and immunologically distinct from the 270 kDa CD36-recognition ligand, sequestrin, on the surface of IRBC which bind only to CD36 (Ockenhouse *et al.*, *J. Infect. Dis. 164*:163-169 (1991); Ockenhouse *et al.*, *Proc. Natl. Acad. Sci. USA 88*:3175-3179 (1991)). Furthermore, the inhibition of IRBC binding to ICAM-1 by soluble ICAM-1 or synthetic peptides provides a therapeutic use for ICAM-1 analogues in severe and complicated malaria which spare important adhesive interactions between ICAM-1 and its counter-receptors LFA-1 and Mac-1.

TABLE 1

**Adhesion of Plasmodium falciparum-infected erythrocytes to deletion mutant ICAM-1 and ICAM-2 molecules expressed in COS cells**

| | | Malaria-infected Erythrocytes (IRBC Bound / 100 COS cells) | | | |
|---|---|---|---|---|---|
| COS Cell Transfectants | LFI | ItG-ICAM-1 | ItG-CD36 | CY25 | G15 |
| Wild-type | 14 | 2176 ± 258 | 64 ± 11 | 2203 ± 432 | 706 ± 190 |
| MOCK | -- | 22 ± 8 | 30 ± 14 | 29 ± 14 | 21 ± 14 |
| $D3^-$ | 3 | 903 ± 220 | 23 ± 13 | 1618 ± 351 | 600 ± 79 |
| $D4^-$ | 11 | 1222 ± 307 | 43 ± 35 | 1383 ± 185 | 638 ± 122 |
| $D4^-D5^-$ | 11 | 1293 ± 229 | 44 ± 24 | 1290 ± 193 | 515 ± 127 |
| ICAM-2 | 21 | 23 ± 7 | 45 ± 21 | 35 ± 18 | 4 ± 11 |

Laboratory-adapted (ItG) and naturally-acquired (CY25, G15) IRBC were assayed for adhesion to COS cells transfected with cDNAs from wild-type ICAM-1 and ICAM-2 or ICAM-1 domain deletion mutants as described in Experimental Procedures. The linear fluorescence intensity (LFI) of ICAM-1 or ICAM-2 monoclonal antibody binding to transfected COS cells was determined by indirect immunofluorescence and analyzed by flow cytometry. Results represent the mean ± standard divisions of three determinations.

Note: $D3^-$ always expressed 1/2 that of other deletions.

TABLE 2

Adhesion of *Plasmodium falciparum*-infected erythrocytes to ICAM-1 amino acid substitution mutants expressed in COS cells

| Mutation Domain 1 | IRBC Binding (% wt ± sd) | LFA-1 Binding (% wt ± sd) | HRV14 Binding (% wt ± sd) |
|---|---|---|---|
| QIT/KA | 102 ± 13 | | |
| Q1/E | 146 ± 12 | | |
| S3/T | 126 ± 8 | | |
| S5/T | 137 ± 29 | | |
| S7KV/RKV | 102 ± 20 | 71 ± 23 | 103 ± 6 |
| K8/E | 138 ± 12 | | |
| R13/K | 120 ± 7 | | |
| R13G/EA | 6 ± 6 | | |
| G15S/SA | 2 ± 0.2 | | |
| L18/Q | 15 ± 3 | 132 ± 26 | 126 ± 22 |
| T20CS/ACT | 118 ± 6 | | |
| S24/A | 127 ± 37 | | |
| D26QPK/ALPE | 135 ± 5 | * | * |
| E34/A | 109 ± 19 | * | * |
| L37/S | 102 ± 18 | 82 ± 19 | 39 ± 3 |
| K39KE/ERQ | 88 ± 15 | | * |
| K40/A | 116 ± 5 | | |
| L43LPGN/RLPG | 109 ± 17 | 142 ± 38 | 145 ± 13 |
| G46NN/ASI | 69 ± 17 | * | * |
| N48/H | 122 ± 22 | | |
| R49KV/EKL | 81 ± 11 | | * |
| Y52/F | 117 ± 15 | | |
| Y52E/FA | 104 ± 22 | | |
| N56V/HM | 110 ± 13 | | |
| Q58/H | 98 ± 13 | | |
| E59/K | 97 ± 17 | | |
| D60/N | 100 ± 19 | | |
| D60S/KL | 2 ± 1 | | |
| S61/I | 95 ± 15 | | |
| Q62PM/API | 97 ± 28 | | |
| Y66/T | 124 ± 20 | | |
| N68/K | 106 ± 26 | | |
| D71/E | 85 ± 15 | | |

TABLE 2

Adhesion of *Plasmodium falciparum*-infected erythrocytes to ICAM-1 amino acid substitution mutants expressed in COS cells

| Mutation Domain 1 | IRBC Binding (% wt ± sd) | LFA-1 Binding (% wt ± sd) | HRV14 Binding (% wt ± sd) |
|---|---|---|---|
| D71/N | 109 ± 12 | | |
| Q73/T | 123 ± 24 | * | |
| S74/A | 98 ± 26 | | |
| T75/A | 106 ± 30 | | |
| K77T/ES | 80 ± 6 | | |
| T78F/SL | 122 ± 13 | 137 ± 46 | 142 ± 54 |
| R88V/EA | 100 ± 11 | | |
| E90/Q | 105 ± 17 | | |
| Domain 2 | | | |
| G101K/AN | 94 ± 6 | | |
| E111GGA/KAGS | 98 ± 23 | | |
| N118/Q | 127 ± 26 | | |
| R125E | 97 ± 6 | | |
| E127/R | 96 ± 20 | | |
| K128/R | 90 ± 14 | | |
| V136GE/GVK | 100 ± 9 | | |
| R149RD/EEG | 130 ± 20 | | |
| H152HGA/EEGS | 121 ± 19 | | |
| N156/E | 150 ± 15 | | |
| R166PQ/EPA | 107 ± 13 | | |

TABLE 2

Adhesion of *Plasmodium falciparum*-infected erythrocytes to ICAM-1 amino acid substitution mutants expressed in COS cells

| Mutation Domain 1 | IRBC Binding (% wt ± sd) | LFA-1 Binding (% wt ± sd) | HRV14 Binding (% wt ± sd) |
|---|---|---|---|
| N175/A | 98 ± 29 | | |
| S177/G | 117 ± 12 | | |

ICAM-1 amino acid substitution mutants were generated by oligonucleotide-directed mutagenesis (Staunton *et al.*, *Cell 61*:243-254 (1990)). Wild-type (wt) residues precede the slash and are followed by the substitution residues in the mutant. IRBC adhesion to COS cells expressing mutant ICAM-1 was assessed by concurrent monoclonal antibody CL203 staining and IRBC adhesion and expressed as the mean percentage ± standard deviation (sd) binding of IRBC to wild-type ICAM-1 transfected cells. The values for LFA-1 binding and HRV14 binding to the new mutants generated for these studies are shown in the columns within the table. *Amino acid substitution mutants with decreased binding as previously published (Staunton *et al.*, *Cell 61*:243-254 (1990)).

## Example 5

### Chimeric proteins consisting of soluble ICAM-1 or soluble CD36 and an Antibody Fragment

Since adhesion of IRBC to microvascular endothelium is an absolute requirement for survival of *P. falciparum* parasites *in vivo* (Howard *et al., Blood 74*:2603-2618 (1989)), a strategy was fashioned to both inhibit infected erythrocyte adhesion and kill the intracellular parasite. We designed an immunoadhesin consisting of the first two $NH_2$-terminal immunoglobulin-like domains of ICAM-1 or CD36 deleted for the transmembrane spanning region fused to the hinge region and $CH_2$ and $CH_3$ domains of human IgG1 heavy chain and expressed it in COS cells (Fig. 1a). The secreted mature molecule designated F185G1 (ICAM-1) exists as a dimer migrating at 140,000 $M_r$ when not reduced and 70,000 $M_r$ when reduced (Fig. lb). These sizes agree with that predicted for F185G1. The secreted mature molecule D30F429 (CD36) exists as protein migrating at 120,000 $M_r$ when reduced. This size agrees with that predicted for D30F429.

Other immunoadhesin based on CD36 (CDBG1) have been rationally designed or generated and expressed as described earlier. These include Q40G435, G46G435, Q34V433, Q40V433, F50V433, D30L439, Q34L439, and Q40L439. Each of the CD36 immunoadhesins can have its collagen binding ability deleted using the methods described earlier.

The adhesion of IRBC to F185G1 immunoadhesin was compared to that of a soluble form of ICAM-1 (sICAM-1) possessing all 5 Ig-like domains that was produced in CHO cells (Marlin *et al., Nature 344*:70-72 (1990)) or insect cells (Diamond *et al., Cell 65*:961-971 (1991)). Malaria-infected erythrocytes bind in a dose-dependent manner to sICAM-1 and F185G1 coated on surfaces (Fig. 2a). The immunoadhesin did not bind uninfected erythrocytes nor erythrocytes infected with malaria parasites which bind to an alternative endothelial receptor, CD36 (data not shown).

The ICAM-1 immunoadhesin is a more effective inhibitor of IRBC adhesion to ICAM-1-coated surface than sICAM-1 (Fig. 2b). Fifty percent inhibition of IRBC binding is achieved with approximately 8 fold less F185G1 than sICAM-1. Enhanced binding may reflect the multivalent nature of F185G1.

The adhesion of T-lymphoblastoid cells (SKW-3) to F185G1 was characterized and compared to that of IRBC binding to CD36. SKW-3 cells adhere to F185G1 on a solid substrate and binding is enhanced by PMA-induced activation of LFA-1 (Fig. 2c). Concentrations of soluble F185G1 completely block IRBC binding do not inhibit LFA-1 dependent SKW-3 binding to sICAM-1 coated surfaces (Fig. 2d). In addition binding of soluble F185G1 to lymphoblastoid cells with or without PMA treatment can not be detected by indirect immunofluorescence (data not presented). Hence the avidity of F185G1 is higher for the receptor on IRBC than for LFA-1.

We tested the ability of the ICAM-1 immunoadhesin to support phagocytosis of IRBC. The Fc region of IgG1 was chosen for the immunoadhesin because this subclass is the most effective in triggering antibody-dependent cellular cytotoxicity (Riechmann *et al., Nature* 332:323-327 (1988)) and binds avidly to all three classes of Fcγ receptor (Unkeless *et al., Annu. Rev. Immunol.* 6:251-281 (1988)). Incubation of parasitized erythrocytes that bind to ICAM-1 (ItG-ICAM IRBC) with the F185G1 chimera resulted in their phagocytosis suggesting that the FcR binding function of F185G1 is intact (Fig. 3). Infected erythrocytes incubated with or without normal human IgG were not phagocytosed. CD36-binding IRBC incubated in the presence or absence of F185G1 chimera were not phagocytosed. The F185G1-treated internalized IRBC are quickly degraded and residual parasite-derived hemozoin pigment observed intracellularly (Fig. 4b,c). CD36-binding IRBC attach to CD36 on the surface of monocytes but are not phagocytized through this receptor (Fig 4a). The rosetting of ItG-CD36 IRBC with monocytes was blocked completely by the anti-CD36 monoclonal antibody OKM5 (data not shown). The ICAM-1-binding IRBC are not rosetted or phagocytosed

in the absence of F185G1 (Fig. 4d). We have designed an ICAM-1 immunoadhesin and a CD36 immunoadhesin that is effective against *P. falciparum* parasitized erythrocytes but does not block lymphocytic binding to ICAM-1. Sequestration of *P. falciparum* IRBC plays a pivotal role in the pathology of malaria, probably by triggering a cascade of deleterious events including local anoxia, induction of toxic inflammatory mediators, edema and tissue damage. Sequestration in the brain leads to the most fatal form of the disease, cerebral malaria (World Health Organization Malaria Action Programme, *Trans. R. Soc. Trop. Med. Hyg. 80* Suppl.:3-50 (1986)). Immunoadhesins mimicking *P. falciparum* sequestration receptors can be therapeutically effective through two distinct mechanisms. First, they should reverse sequestration; a combination of adhesins, including ICAM-1 and CD36 immunoadhesin, may be required for maximal effectiveness. Reversal of sequestration is predicted to alleviate much of the associated pathology and especially mortality resulting from cerebral malaria or placental insufficiency. Second, immunoadhesins can sensitize parasitized erythrocytes for recognition and elimination by the immune system, as exemplified here by monocyte phagocytosis and destruction mediated by an ICAM-1 immunoadhesin. Release from sequestration is not necessarily required for this effector mechanism, as it could presumably be mediated by monocytes and granulocytes at sites of sequestration in post capillary venules. A side benefit of clearance of parasites by phagocytes is that it boosts host humoral and cellular immunity to *P. falciparum*. Cytoadherence receptor binding must be conserved and thus pathogen strain variation, which is extensive for *P. falciparum*, would not be an effective mechanism for evasion of this therapy.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: STAUNTON, DONALD E
SPRINGER, TIMOTHY A

(ii) TITLE OF INVENTION: CD 36 IMMUNOADHESINS, AND THEIR USE IN SELECTIVELY KILLING PLASMODIUM FALCIPARUM INFECTED ERYTHROCYTES

(iii) NUMBER OF SEQUENCES: 14

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Sterne, Kessler, Goldstein & Fox
(B) STREET: 1225 Connecticut Ave. NW Suite 300
(C) CITY: Washington
(D) STATE: D.C.
(E) COUNTRY: USA
(F) ZIP: 20036

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/862,708
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(B) FILING DATE: 03-OCT-1991

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: FOX, SAM L
(B) REGISTRATION NUMBER: 30,353
(C) REFERENCE/DOCKET NUMBER: 1011.0610003

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (202) 466-0800
(B) TELEFAX: (202) 833-8716

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Ser Val Leu Val Thr
1 5

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Gln Thr Ser Val Ser Pro Ser Lys Val Ile Leu Pro Arg Gly Gly Ser
1 5 10 15

Val Leu Val Thr Cys Ser Thr Ser Cys Asp Gln Pro Lys Leu Xaa Leu
20 25 30

Gly Ile Glu Thr Pro Leu Pro
35

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gln Val Ser Ile His Pro Arg Glu Ala Phe Leu Pro Gln Gly Gly Ser
1 5 10 15

Val Gln Val Asn Cys Ser Ser Ser Cys Lys Glu Xaa Asp Leu Ser Leu
20 25 30

Gly Leu Glu Thr Gln Trp Leu
35

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 39 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu Val His Val Arg Pro Lys Lys Leu Ala Val Glu Pro Lys Gly Ser
1 5 10 15

Leu Glu Val Asn Cys Ser Thr Thr Cys Asn Gln Pro Glu Val Xaa Gly
20 25 30

Gly Leu Glu Thr Ser Leu Xaa
35

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Lys Lys Glu Leu Leu Leu Pro Gly Asn Asn Arg Lys Val Tyr Glu Leu
1 5 10 15

Ser Asn Val Gln Glu Asp Ser Gln Pro Met Cys Tyr Ser Asn Cys Pro
20 25 30

Asp Gly Gln Ser Thr Ala Lys Thr
35 40

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Lys Asp Glu Leu Glu Xaa Ser Gly Pro Asn Trp Lys Leu Phe Glu Leu
1 5 10 15

Ser Glu Ile Gly Glu Asp Ser Ser Pro Leu Cys Phe Glu Asn Cys Gly
20 25 30

Thr Val Gln Ser Ser Ala Ser Ala
35 40

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 40 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Lys Ile Leu Leu Asp Glu Gln Ala Gln Trp Lys His Tyr Leu Val
1 5 10 15

Ser Asn Ile Ser His Asp Thr Val Leu Gln Cys His Phe Thr Cys Ser
20 25 30

Gly Lys Gln Glu Ser Met Asn Ser
35 40

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Gly Gly Ser Val Leu Val
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Val Leu Val Thr
1

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

TTTCTCGAGG GTGTCTGCTG GAAGCAGGCT CAG 33

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

TTTGCGGCCG CTGGGAGCGG GGCTTGCCGG CCGTCG 36

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ACCGGAAGCT TCTAGAGATC CCTCGACCAC GAGATCCATT GTGC 44

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(D) TOPOLOGY: both

(ii) MOLECULE TYPE: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TTCTGAGTCT CACCAAAGGT CTGGAGCTGG TAGGGGGC 38

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 471 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Gly Cys Asp Arg Asn Cys Gly Leu Ile Ala Gly Ala Val Ile Gly Ala
1 5 10 15

Val Leu Ala Val Phe Gly Gly Ile Leu Met Pro Val Gly Asp Leu Leu
20 25 30

Ile Gln Lys Thr Ile Lys Lys Gln Val Val Leu Glu Glu Gly Thr Ile
35 40 45

Ala Phe Lys Asn Trp Val Lys Thr Gly Thr Glu Val Tyr Arg Gln Phe
50 55 60

Trp Ile Phe Asp Val Gln Asn Pro Gln Glu Val Met Met Asn Ser Ser
65 70 75 80

Asn Ile Gln Val Lys Gln Arg Gly Pro Tyr Thr Tyr Arg Val Arg Phe
85 90 95

Leu Ala Lys Glu Asn Val Thr Gln Asp Ala Glu Asp Asn Thr Val Ser
100 105 110

Phe Leu Gln Pro Asn Gly Ala Ile Phe Glu Pro Ser Leu Ser Val Gly
115 120 125

Thr Glu Ala Asp Asn Phe Thr Val Leu Asn Leu Ala Val Ala Ala Ala
130 135 140

Ser His Ile Tyr Gln Asn Gln Phe Val Gln Met Ile Leu Asn Ser Leu
145 150 155 160

Ile Asn Lys Ser Lys Ser Ser Met Phe Gln Val Arg Thr Leu Arg Glu
165 170 175



SUBSTITUTE SHEET

Leu Leu Trp Gly Tyr Arg Asp Pro Phe Leu Ser Leu Val Pro Tyr Pro
180 185 190

Val Thr Thr Thr Val Gly Leu Phe Tyr Pro Tyr Asn Asn Thr Ala Asp
195 200 205

Gly Val Tyr Lys Val Phe Asn Gly Lys Asp Asn Ile Ser Lys Val Ala
210 215 220

Ile Ile Asp Thr Tyr Lys Gly Lys Arg Asn Leu Ser Tyr Trp Glu Ser
225 230 235 240

His Cys Asp Met Ile Asn Gly Thr Asp Ala Ala Ser Phe Pro Pro Phe
245 250 255

Val Glu Lys Ser Gln Val Leu Gln Phe Phe Ser Ser Asp Ile Cys Arg
260 265 270

Ser Ile Tyr Ala Val Phe Glu Ser Asp Val Asn Leu Lys Gly Ile Pro
275 280 285

Val Tyr Arg Phe Val Leu Pro Ser Lys Ala Phe Ala Ser Pro Val Glu
290 295 300

Asn Pro Asp Asn Tyr Cys Phe Cys Thr Glu Lys Ile Ile Ser Lys Asn
305 310 315 320

Cys Thr Ser Tyr Gly Val Leu Asp Ile Ser Lys Cys Lys Glu Gly Arg
325 330 335

Pro Val Tyr Ile Ser Leu Pro His Phe Leu Tyr Ala Ser Pro Asp Val
340 345 350

Ser Glu Pro Ile Asp Gly Leu Asn Pro Asn Glu Glu Glu His Arg Thr
355 360 365

Tyr Leu Asp Ile Glu Pro Ile Thr Gly Phe Thr Leu Gln Phe Ala Lys
370 375 380

Arg Leu Gln Val Asn Leu Leu Val Lys Pro Ser Glu Lys Ile Gln Val
385 390 395 400

Leu Lys Asn Leu Lys Arg Asn Tyr Ile Val Pro Ile Leu Trp Leu Asn
405 410 415

Glu Thr Gly Thr Ile Gly Asp Glu Lys Ala Asn Met Phe Arg Ser Gln
420 425 430

Val Thr Gly Lys Ile Asn Leu Leu Gly Leu Ile Glu Met Ile Leu Leu
435 440 445

Ser Val Gly Val Val Met Phe Val Ala Phe Met Ile Ser Tyr Cys Ala
450 455 460

Cys Arg Ser Lys Thr Ile Lys
465 470

WHAT IS CLAIMED IS:

1. A method of blocking the binding of an IRBC to CD36 which comprises contacting said IRBC with a therapeutically effective amount of an agent capable of binding to the CD36 binding site on the IRBC wherein said agent is an antibody derivative of CD36, or an antibody derivative of a fragment of CD36.

2. A method for selectively killing an IRBC which comprises providing to an IRBC an effective amount of an antibody-derivatized agent, said antibody-derivatized agent comprising an antibody or fragment thereof covalently attached to CD36, or a fragment thereof.

3. The method of claims 1 or 2 wherein said CD36, or fragment thereof is additionally incapable of binding to collagen.

4. The method of claims 1 or 2 wherein said CD36 fragment is CD36 deleted for one or more regions selected from the group consisting of residues 1-5, 6-28, 439-465 and 466-471.

5. The method of claims 1 or 2 wherein said fragment of CD36 begins with an amino acid residue selected from the group consisting of D30, Q34, Q40, G46, or F50 of CD36 and continues to an amino acid residue selected from the group consisitng of N416, F429, V433, G435, or L439 of CD36.

6. The method of claims 1 or 2 wherein said agent is provided to a patient in need of such a treatment in a therapeutically effective amount.

7. The method of claims 1 or 2 wherein said agent is administered by enteral means, parenteral means, inhalation means

intranasal means or transdermal means.

8. The method of claims 1 or 2 wherein said agent is administered prophylactically.

9. The method of claims 1 or 2 wherein said agent is administered therapeutically.

10. The method of claim 8 wherein said parenteral means is intramuscular, intravenous or subcutaneous.

11. A pharmaceutical composition comprising an antibody-derivative of CD36, or an antibody derivative of a fragment of CD36.

12. A diagnostic composition comprising an agent of claim 11 in a detectably labelled form.

13. A diagnostic composition comprising an agent of claim 11 in an immobilized form.

14. The pharmaceutical composition of claim 11 wherein said fragment of CD36 is CD36 delete for one or more regions selected from the group consisting of residues 1-5, 6-28, 439-465 and 466-471.

15. The pharmaceutical composition of claim 11 wherein said fragment of CD36 begins with an amino acid residue selected from the group consisting of D30, Q34, Q40, G46, or F50 of CD36 and continues to an amino acid residue selected from the group consisitng of N416, F429, V433, G435, or L439 of CD36.

16. An antibody derivative of a peptide capable of binding to the CD36 binding site on an IRBC.

17. The antibody-derivative of claim 16 wherein said peptide is CD36, or a fragment thereof, and said fragment of said CD36 begins with an amino acid residue selected from the group consisting of D30, Q34, Q40, G46, or F50 of CD36 and continues to an amino acid residue selected from the group consisitng of N416, F429, V433, G435, or L439 of CD36.

18. The antibody derivative of claim 16 wherein said peptide is CD36 delete for one or more regions selected from the group consisting of residues 1-5, 6-28, 439-465 and 466-471.

19. A DNA sequence capable of encoding any one of said antibody derivatives of any one of claims 16-18.

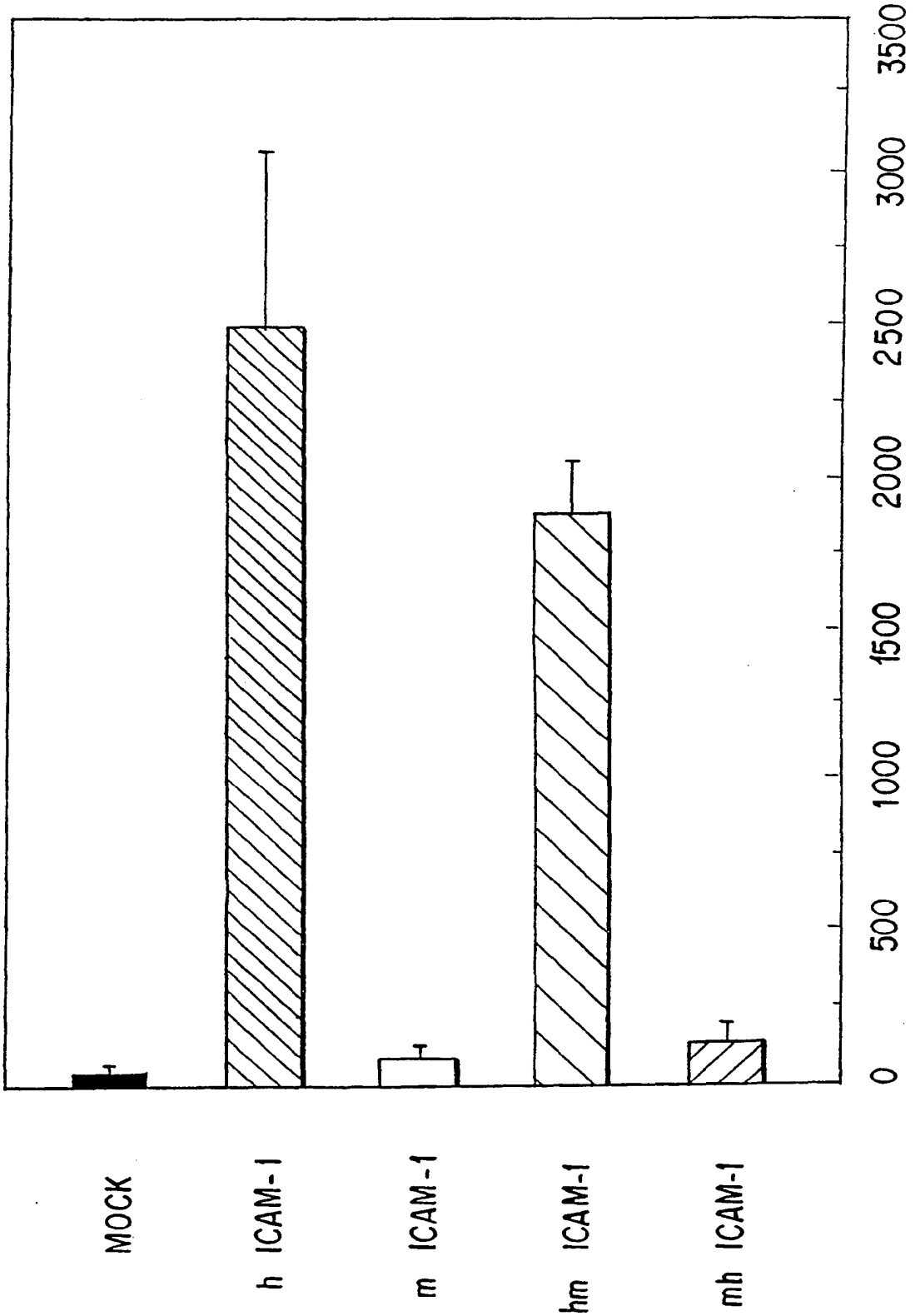

MALARIA-INFECTED ERYTHROCYTES BOUND
0
500
1000
1500
2000
2500
3000
3500
MOCK
h ICAM-1
m ICAM-1
hm ICAM-1
mh ICAM-1

FIG. 1

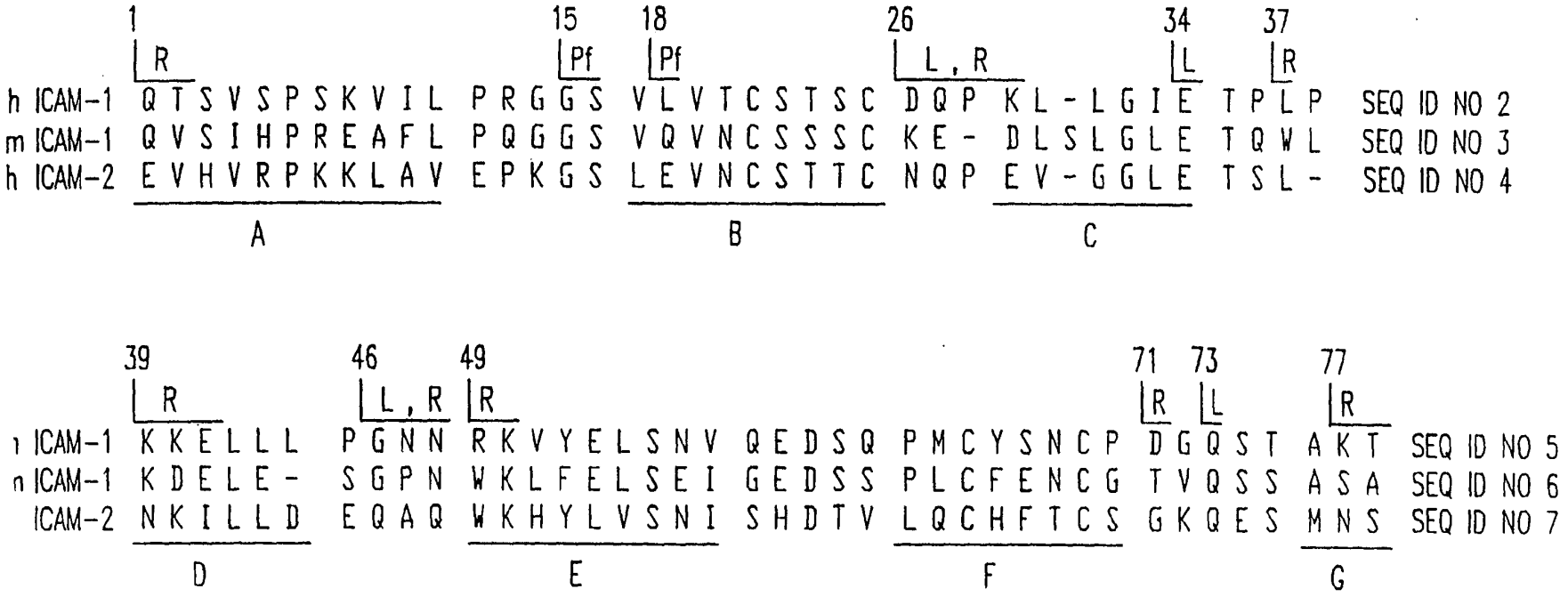



FIG.2

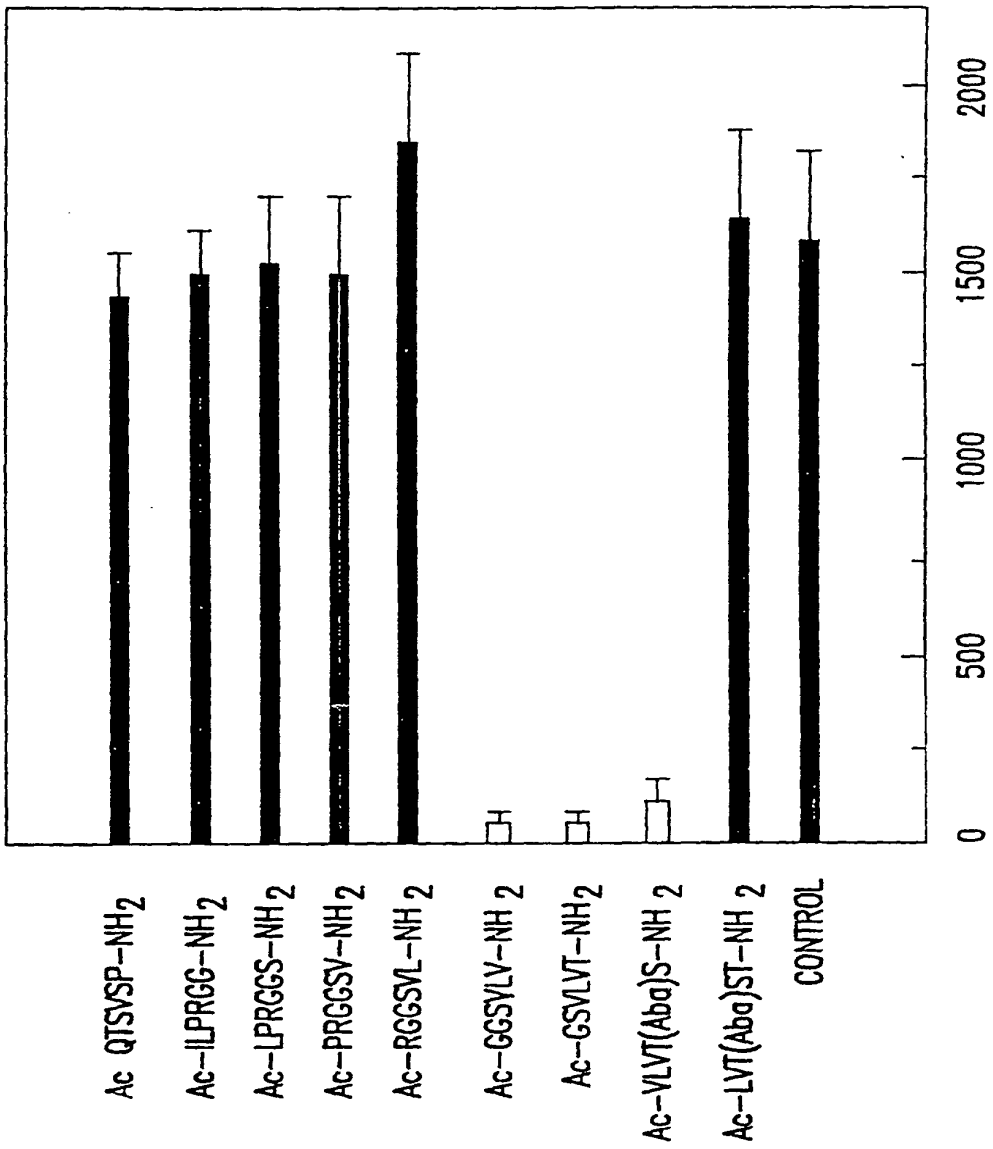



FIG. 3A

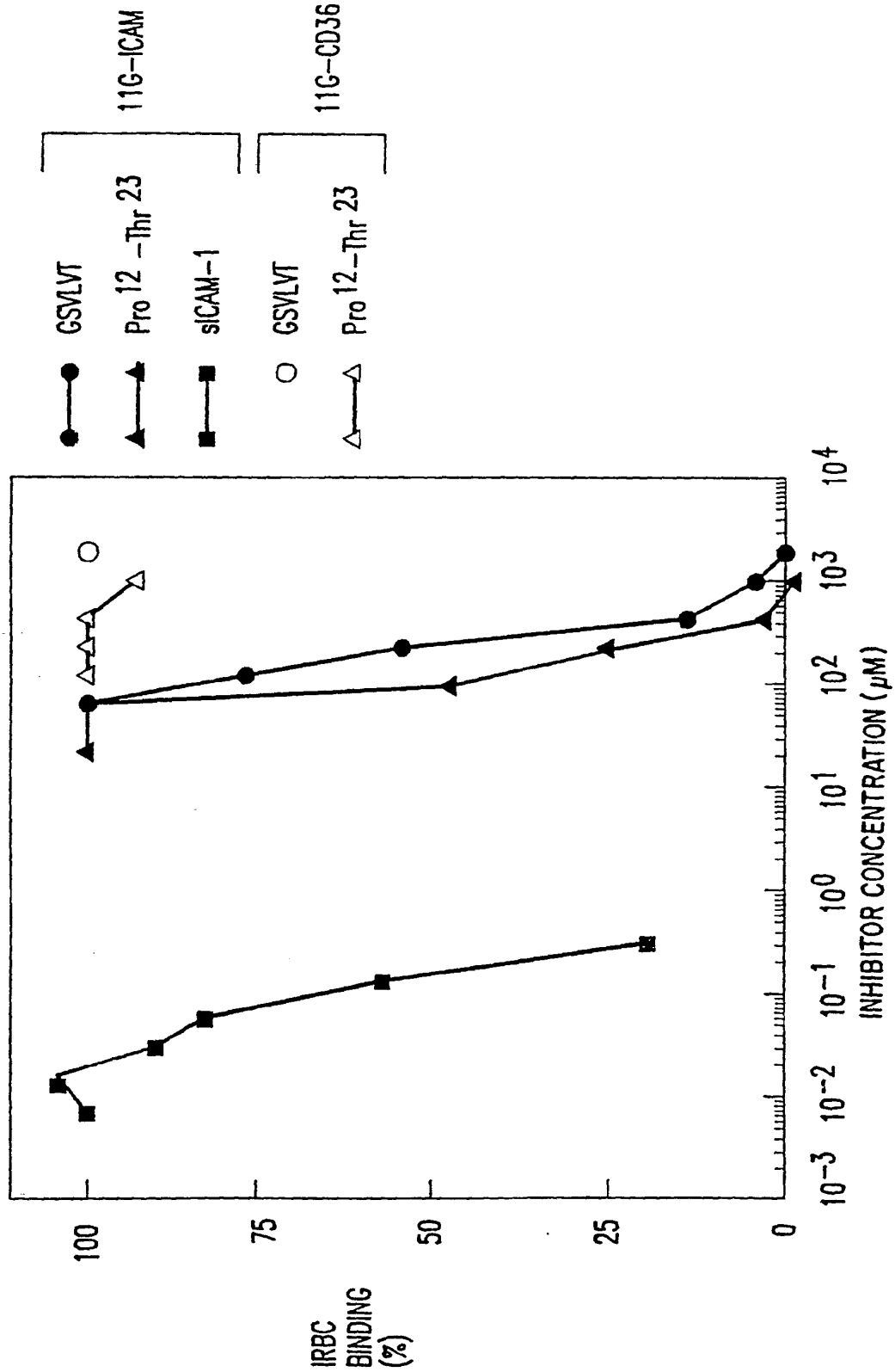
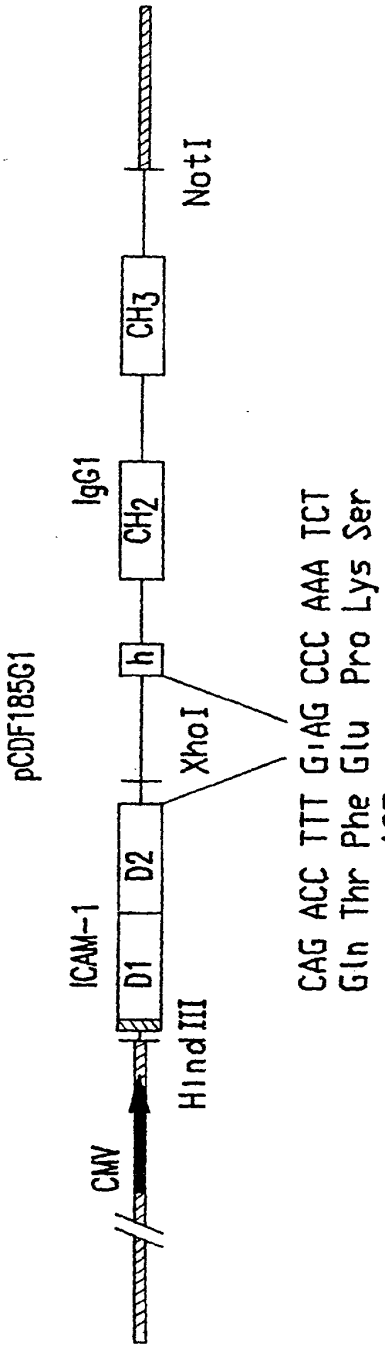



FIG.3B


pCDF185G1
CMV
HindIII
ICAM-1
D1
D2
XhoI
h
IgG1
CH2
CH3
NotI
CAG ACC TTT GtAG CCC AAA TCT
Gln Thr Phe Glu Pro Lys Ser
185

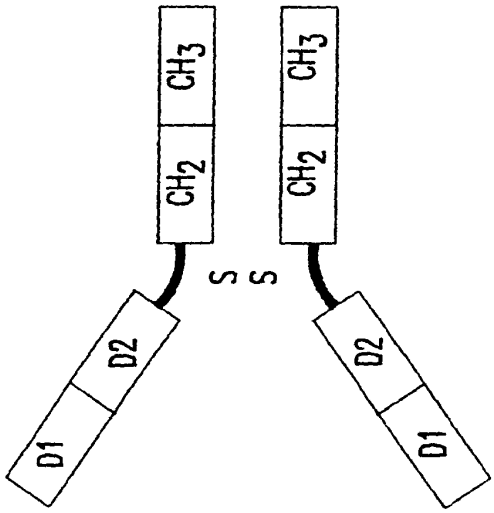

CH3
CH2
D2
D1
S
S
F185G1

FIG 4A

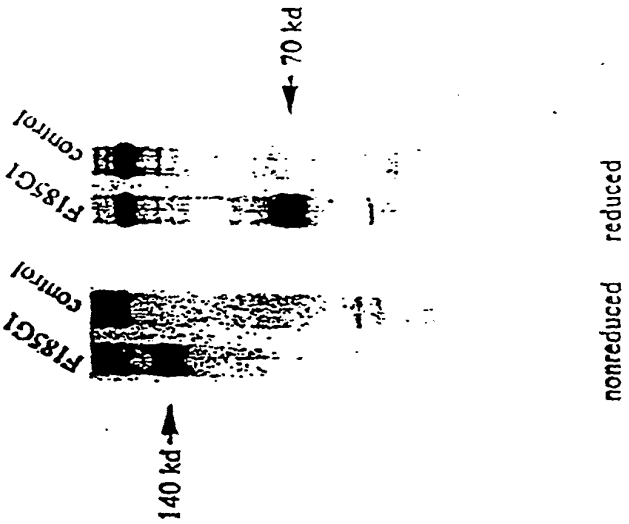



Figure 4B

Figure 1B

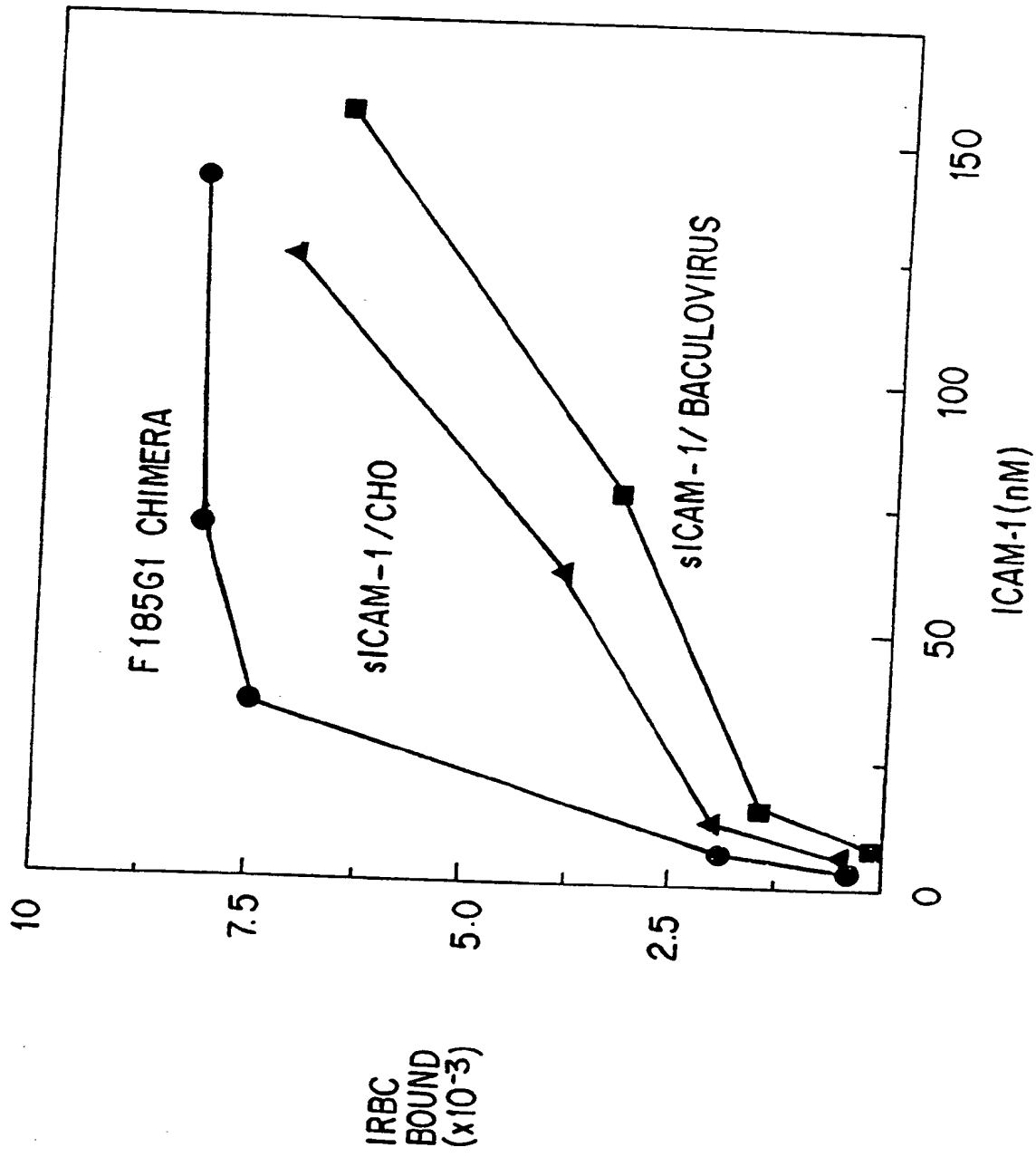

F185G1 CHIMERA
sICAM-1/CHO
sICAM-1/BACULOVIRUS
IRBC BOUND (x10-3)
10
7.5
5.0
2.5
0
50
100
150
ICAM-1(nM)

FIG.5A

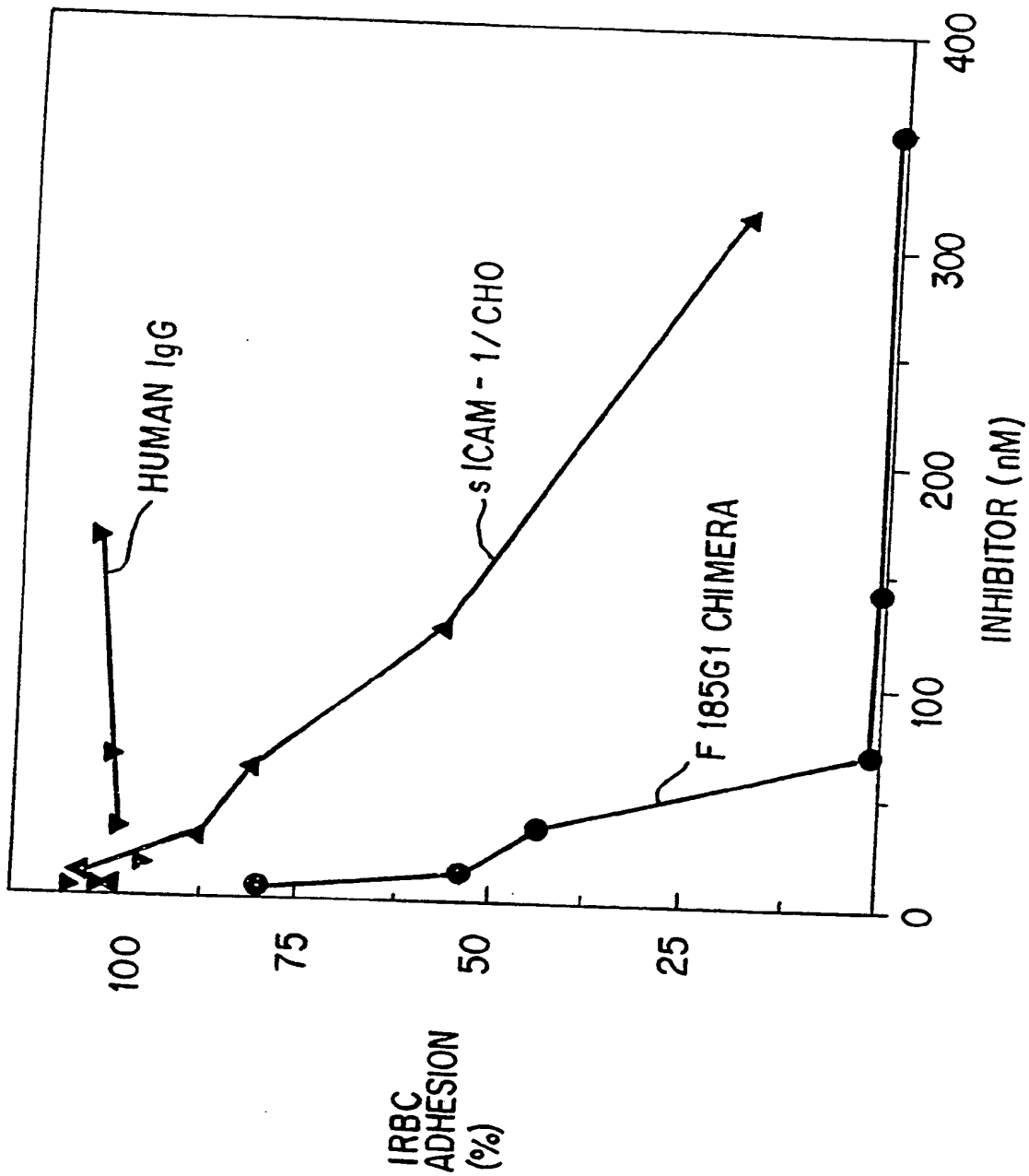



FIG. 5B

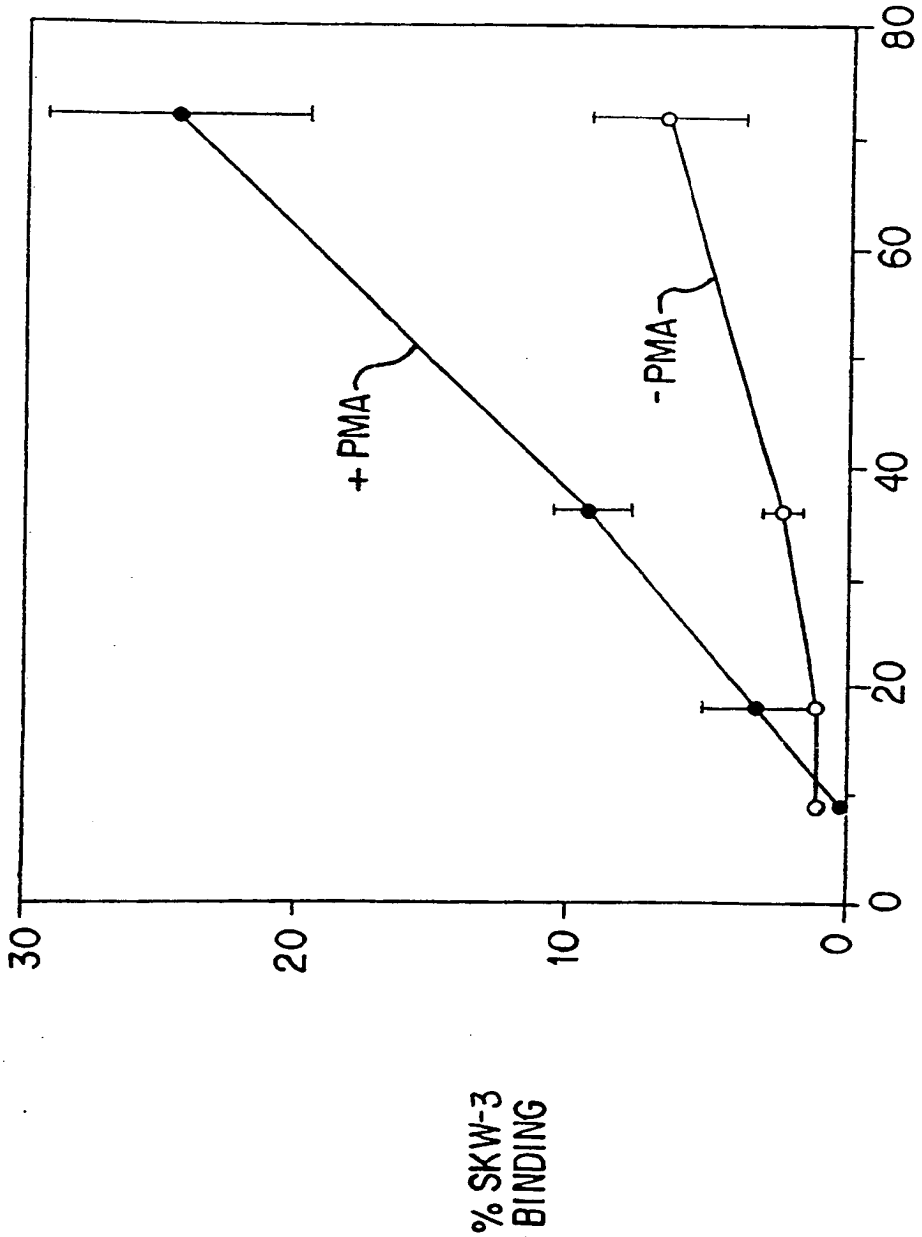



FIG. 5C



SUBSTITUTE SHEET

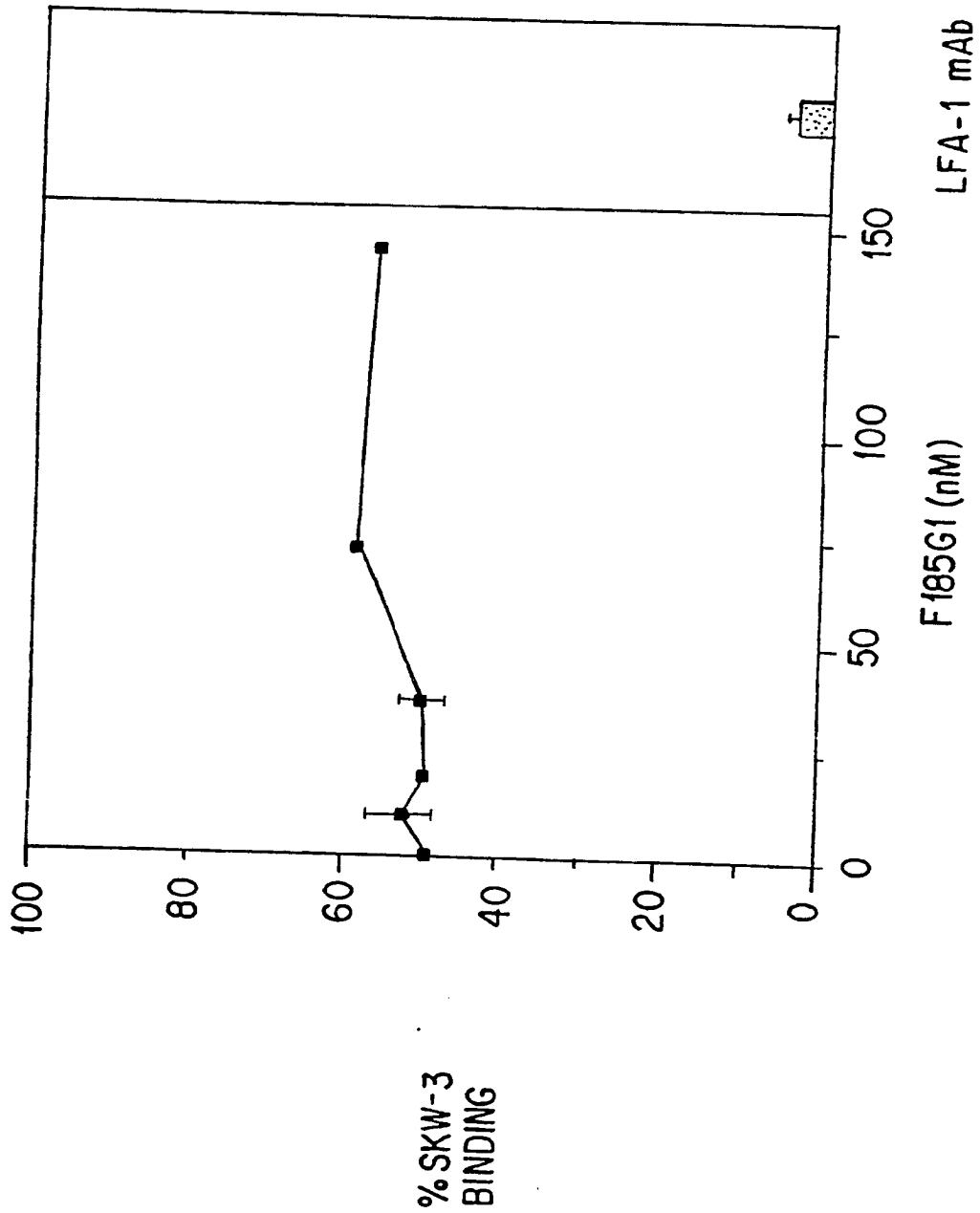



FIG. 5D

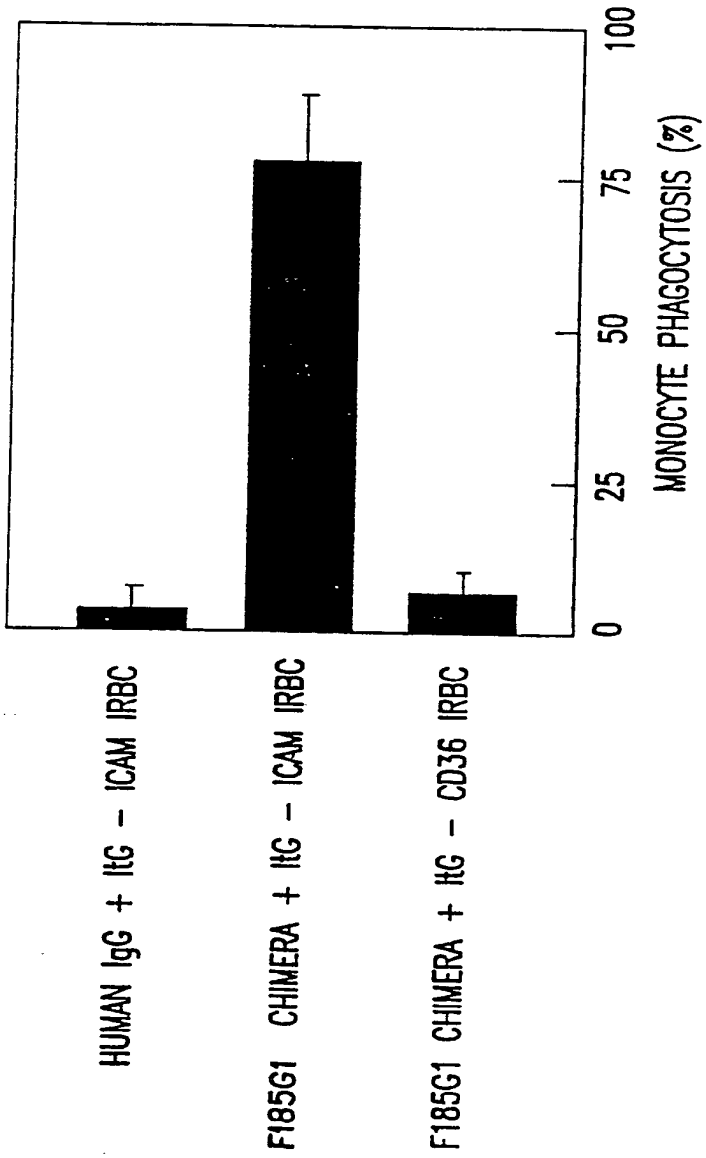
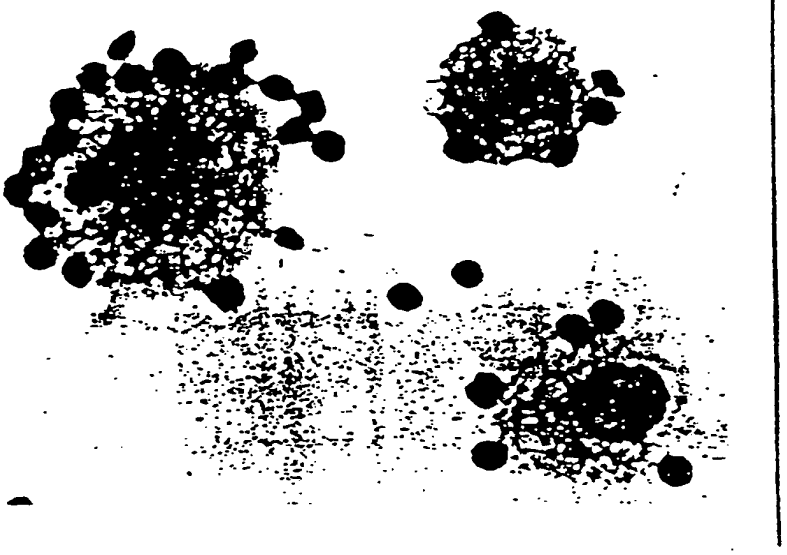



FIG.6



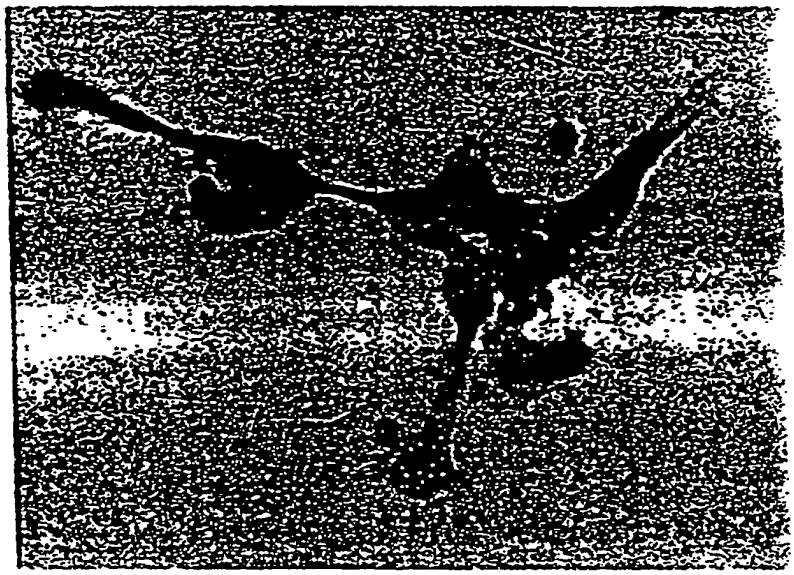


Figure 7A-B

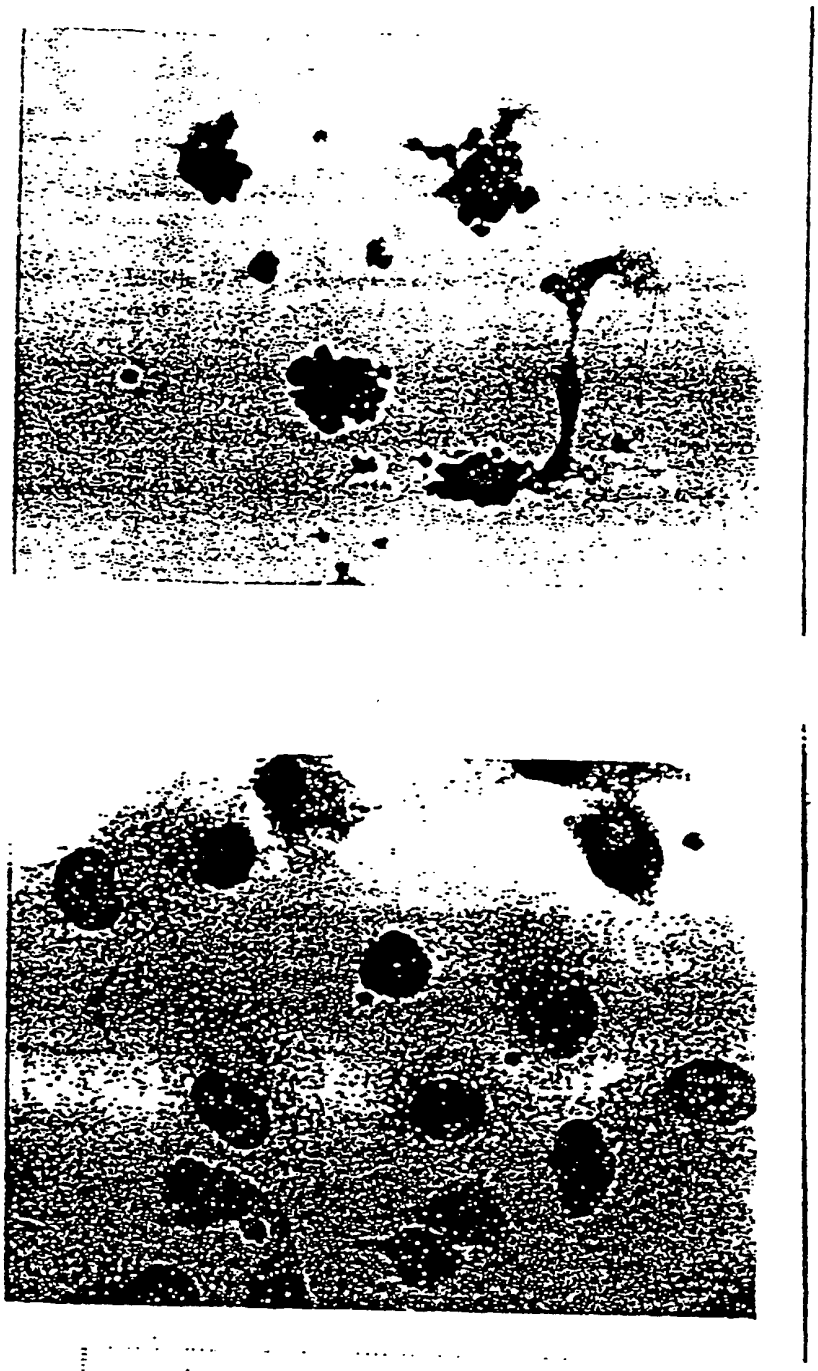


Figure 7C-D

Gly Cys Asp Arg Asn Cys Gly Leu Ile Ala Gly Ala Val Ile Gly Ala
1 5 10 15

Val Leu Ala Val Phe Gly Gly Ile Leu Met Pro Val Gly Asp Leu Leu
20 25 30

Ile Gln Lys Thr Ile Lys Lys Gln Val Val Leu Glu Glu Gly Thr Ile
35 40 45

Ala Phe Lys Asn Trp Val Lys Thr Gly Thr Glu Val Tyr Arg Gln Phe
50 55 60

Trp Ile Phe Asp Val Gln Asn Pro Gln Glu Val Met Met Asn Ser Ser
65 70 75 80

Asn Ile Gln Val Lys Gln Arg Gly Pro Tyr Thr Tyr Arg Val Arg Phe
85 90 95

Leu Ala Lys Glu Asn Val Thr Gln Asp Ala Glu Asp Asn Thr Val Ser
100 105 110

Phe Leu Gln Pro Asn Gly Ala Ile Phe Glu Pro Ser Leu Ser Val Gly
115 120 125

Thr Glu Ala Asp Asn Phe Thr Val Leu Asn Leu Ala Val Ala Ala Ala
130 135 140

Ser His Ile Tyr Gln Asn Gln Phe Val Gln Met Ile Leu Asn Ser Leu
145 150 155 160

Ile Asn Lys Ser Lys Ser Ser Met Phe Gln Val Arg Thr Leu Arg Glu
165 170 175

Leu Leu Trp Gly Tyr Arg Asp Pro Phe Leu Ser Leu Val Pro Tyr Pro
180 185 190

Val Thr Thr Thr Val Gly Leu Phe Tyr Pro Tyr Asn Asn Thr Ala Asp
195 200 205

FIG. 8A

Gly Val Tyr Lys Val Phe Asn Gly Lys Asp Asn Ile Ser Lys Val Ala
210 215 220

Ile Ile Asp Thr Tyr Lys Gly Lys Arg Asn Leu Ser Tyr Trp Glu Ser
225 230 235 240

His Cys Asp Met Ile Asn Gly Thr Asp Ala Ala Ser Phe Pro Pro Phe
245 250 255

Val Glu Lys Ser Gln Val Leu Gln Phe Phe Ser Ser Asp Ile Cys Arg
260 265 270

Ser Ile Tyr Ala Val Phe Glu Ser Asp Val Asn Leu Lys Gly Ile Pro
275 280 285

Val Tyr Arg Phe Val Leu Pro Ser Lys Ala Phe Ala Ser Pro Val Glu
290 295 300

Asn Pro Asp Asn Tyr Cys Phe Cys Thr Glu Lys Ile Ile Ser Lys Asn
305 310 315 320

Cys Thr Ser Tyr Gly Val Leu Asp Ile Ser Lys Cys Lys Glu Gly Arg
325 330 335

Pro Val Tyr Ile Ser Leu Pro His Phe Leu Tyr Ala Ser Pro Asp Val
340 345 350

Ser Glu Pro Ile Asp Gly Leu Asn Pro Asn Glu Glu Glu His Arg Thr
355 360 365

Tyr Leu Asp Ile Glu Pro Ile Thr Gly Phe Thr Leu Gln Phe Ala Lys
370 375 380

Arg Leu Gln Val Asn Leu Leu Val Lys Pro Ser Glu Lys Ile Gln Val
385 390 395 400

Leu Lys Asn Leu Lys Arg Asn Tyr Ile Val Pro Ile Leu Trp Leu Asn
405 410 415

FIG. 8B

Glu Thr Gly Thr Ile Gly Asp Glu Lys Ala Asn Met Phe Arg Ser Gln
420 425 430

Val Thr Gly Lys Ile Asn Leu Leu Gly Leu Ile Glu Met Ile Leu Leu
435 440 445

Ser Val Gly Val Val Met Phe Val Ala Phe Met Ile Ser Tyr Cys Ala
450 455 460

Cys Arg Ser Lys Thr Ile Lys
465 470

FIG. 8C

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US92/08482

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/85.91; 435/4, 7.21, 7.25; 530/387.1, 387.3, 388.2, 388 7, 389.6, 300, 350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS ONLINE, MEDLINE, BIOSIS, EMBASE, APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | Proceedings of the National Academy of Science, U.S.A., Volume 88, issued April 1989, C.F. Ockenhouse et al., "Sequestrin, a CD36 Recognition Protein on Plasmodium Falciparum Malaria-Infected Erythrocytes Identified by Anti-Idiotype Antibodies", pages 3175-3179, see entire document. | 1-18 |
| Y | Cell, Volume 58, issued 14 July 1989, P. Oquendo et al., "CD36 Mediates Cytoadherence of Plasmodium Falciparum Parasitized Erythrocytes", pages 95-101, see entire document. | 1-19 |
| Y | Science, Volume 243, issued 17 March 1989, C.F. Ockenhouse et al., "Identification of a Platelet Membrane Glycoprotein as a Falciparum Malaria Sequestration Receptor", pages 1469-1471, see entire document. | 1-18 |
| Y | Science, Vol. 238, issued 20 November 1987, E.S. Vittetta et al., "Redesigning Nature's Poisons to Create Anti-Tumor Reagents", pages 1098-1104, see entire document. | 1-18 |

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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| "E" | earlier document published on or after the international filing date | "Y" | document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art |
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| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 December 1992 | 15 JAN 1993 |
| Name and mailing address of the ISA/<br>Commissioner of Patents and Trademarks<br>Box PCT<br>Washington, D.C. 20231<br>Facsimile No. NOT APPLICABLE | Authorized officer<br>JACQUELINE G. KRIKORIAN<br>Telephone No. (703) 308-0196 |

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNAT L SEARCH REPORT

national application No.
PCT/US92/08482

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| Y | The Journal of Biological Chemistry, Volume 264, No. 13, issued 05 May 1989, N.N. Tandon et al., "Isolation and Characterization of Platelet Glycoprotein IV (CD36)", pages 7570-7575, see entire document. | 1-18 |
| Y | Journal of Clinical Investigation, Volume 84, issued September 1989, J.W. Barnwell et al., "A Human 88-kD Membrane Glycoprotein (CD36) Functions as a Receptor for Cytoadherence on Plasmodium Falciparum-Infected Erythrocytes", pages 765-772, see entire document. | 1-18 |
| Y | Nature, Volume 339, issued 04 May 1989, A. Traunecker et al., "Highly Efficient Neutralization of HIV with Recombinant CD4-Immunoglobulin Molecules", pages 68-70, see entire document. | 1-19 |
| Y | Nature, Volume 332, issued 24 March 1988, L. Reichmann et al., "Reshaping Human Antibodies for Therapy", pages 323-327, see entire document. | 1-19 |

INTERNATION EARCH REPORT

Inte[redacted]nal application No.
PCT/US92/08482

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5):

A61K 37/02, 39/395, 45/00; C07H 3/00; C07K 15/28; C12N 15/11; G01N 33/50

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

424/85.91; 435/4, 7.21, 7.25; 530/387.1, 387.3, 388.2, 388.7, 389.6, 300, 350; 935/8, 107

**CORRECTED VERSION**

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| A61K 37/02, 39/395, 45/00<br>C07H 3/00, C07K 15/28<br>C12N 15/11, G01N 33/50 | A1 | (43) Internati nal Publication Date: | 15 April 1993 (15.04.93) |

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| 862,708 | 3 April 1992 (03.04.92) | US |
| 899,061 | 12 June 1992 (12.06.92) | US |

(71) Applicant: THE CENTER FOR BLOOD RESEARCH [US/US]; 800 Huntington Avenue, Boston, MA 02115 (US).

(72) Inventors: STAUNTON, Donald, E. ; 124 Chestnut Hill Road, Chestnut Hill, MA 02167 (US). SPRINGER, Timothy, A. ; 28 Monadnock Road, Chestnut Hill, MA 02167 (US).

(74) Agents: FOX, Samuel, L. et al.; Sterne, Kessler, Goldstein & Fox, 1225 Connecticut Avenue, N.W., Suite 300, Washington, DC 20036 (US).

(81) Designated States: AT, AU, BB, BG, BR, CA, CH, CS, DE, DK, ES, FI, GB, HU, JP, KP, KR, LK, LU, MG, MN, MW, NL, NO, PL, RO, RU, SD, SE, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: CD36 IMMUNOADHESINS AND THEIR USE IN SELECTIVELY KILLING *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES

(57) Abstract

The present invention discloses the construction of CD36 immunoadhesin and their use in selectively killing *Plasmodium Falciparum* infected erythrocytes. Disclosed are immunoadhesin containing CD36 or fragments of CD36 deleted for one or more of the regions of residues 1-5, 6-28, 439-465 and 466-471.

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# CD36 IMMUNOADHESINS AND THEIR USE IN SELECTIVELY KILLING *PLASMODIUM FALCIPARUM* INFECTED ERYTHROCYTES

This application is a continuation-in-part of U.S. Serial No. 07/862,708 filed April 3, 1992, which is a continuation-in-part of U.S. Serial No. 07/769,625 filed October 3, 1991.

## FIELD OF THE INVENTION

The present invention relates to agents which bind to the ICAM-1 or the CD36 binding site on malarially infected erythrocytes (IRBC). The present invention additionally relates to molecules capable of binding to the IRBC binding site on ICAM-1 or on CD36. The agents of the present invention include antibodies, peptides, and carbohydrates. These agents are useful in ameliorating the symptoms of malaria since they are capable of inhibiting the binding of an IRBC to either ICAM-1 or CD36 and stimulating the phagocytosis of IRBCs.

The present invention further provides methods for the treatment of malaria, methods of preferentially killing an IRBC, methods of stimulating phagocytosis of an IRBC, and a method of diagnosing the presence of an IRBC.

## BACKGROUND OF THE INVENTION

### Malaria

Erythrocytes infected with the human malaria parasite, *Plasmodium falciparum*, adhere to vascular post-capillary endothelium, and the sequestration of the malaria-infected erythrocytes (IRBC) is a primary event responsible for the clinical complications of severe and cerebral malaria. While immature ring stage parasitized erythrocytes circulate unobstructed throughout the vasculature, adhesion of mature intraerythrocytic stages of the parasite to endothelium averts splenic clearance of IRBC and allows parasite maturation in a microenvironment of low oxygen tension. Two cell surface receptors with broad tissue distribution, intercellular adhesion molecule-1 (ICAM-1, CD54) (Berendt *et al.*, *Nature (Lond.) 341*:57-59 (1989)) and CD36 (GPIV) (Ockenhouse *et al.*, *Science (Wash. D.C.) 243*:1469-1471 (1989)) have recently been identified as endothelial receptors for IRBC. Laboratory-adapted IRBC bind to purified ICAM-1-coated and CD36-coated surfaces and the cytoadherent phenotype of these malaria-infected red cells can be modulated by successive panning on ICAM-1 or CD36-coated surfaces (Ockenhouse *et al.*, *J. Infect. Dis. 164*:163-169 (1991)). Moreover, ICAM-1-specific and CD36-specific monoclonal antibody (MAb) staining of small capillary endothelium from postmortem brain tissue colocalizes with IRBC cytoadherence in patients who have died from complications of cerebral malaria (Barnwell *et al.*, *J. Clin. Invest. 84*:765-772 (1989); Aikawa *et al.*, *Am. J. Trop. Med. Hyg. 43*:30 (1990)).

ICAM-1

ICAM-1, a member of the immunoglobulin-like superfamily, is a monomeric unpaired 90-115 $M_r$ glycoprotein composed of a bent extracellular domain containing five tandemly arranged immunoglobulin-like domains, a transmembrane region, and a cytoplasmic domain (Staunton *et al.*, *Cell 52*:925-933 (1988); Simmons *et al.*, *Nature (Lond.) 331*:624-627 (1988)). ICAM-1 is a ligand for the leukocyte integrins, lymphocyte function antigen-1 (LFA-1; CD11a/CD18) (Rothlein *et al.*, *J. Immunol. 137*:1270-1274 (1986); Marlin *et al.*, *Cell 51*:813-819 (1987)) and Mac-1 (CD11b/CD18) (Diamond *et al.*, *J. Cell. Biol. 111*:3219-3139 (1990); Smith *et al.*, *J. Clin. Invest. 83*:2008-2017 (1989)). The recognition, adhesion, and extravasation of lymphoid and myeloid blood cells through the vascular endothelium is an initial step of host immune response to tissue injury. The CD11/CD18 family of proteins are crucial for leukocyte and myeloid cell adhesion to endothelium, T cell activation, cytotoxic T cell killing, and neutrophil chemotaxis and homotypic aggregation (Larsen *et al.*, *Immunol. Rev. 114*:181 (1990)). ICAM-1 is also subverted as a cellular receptor by the major group of human rhinoviruses (HRV), the etiologic agent of the common cold (Staunton *et al.*, *Cell 56*:849-853 (1989); Greve *et al.*, *Cell 56*:839-847 (1989)). A soluble form of ICAM-1 lacking the transmembrane and cytoplasmic domains binds HRV and inhibits rhinovirus adhesion (Marlin *et al.*, *Nature (Lond.) 344*:70-72 (1990)).

Monoclonal antibody blocking studies have indicated that the binding sites for LFA-1 and HRV are proximal. Analysis of mutant ICAM-1 molecules has demonstrated that mutations in the amino terminal domain have the strongest effect on LFA-1 and HRV binding (Staunton *et al.*, *Cell 61*:243-254 (1990)). Domains D1 and D2

demonstrate a close physical association and appear conformationally linked (Staunton *et al.*, *Cell 52*:925-933 (1988)). Amino acid substitution mutants demonstrate that while the LFA-1 and HRV contact sites overlap, they are distinct (Staunton *et al.*, *Cell 61*:243-254 (1990)). The integrin Mac-1 binds to the third $NH_2$-terminal Ig-like domain of ICAM-1 in contrast to LFA-1 and this binding is influenced by the extent of glycosylation on the ICAM-1 molecule (Diamond *et al.*, *Cell 65*:961-971 (1991)).

The molecular basis for adhesion of malaria-infected erythrocytes to ICAM-1 is not known. Monoclonal antibodies RR1/1 and R6.5 which inhibit binding of LFA-1 and HRV to ICAM-1 have no effect on IRBC binding to purified ICAM-1-coated surfaces (Ockenhouse *et al.*, *J. Infect. Dis. 164*:163-169 (1991)). Recently, we and others have demonstrated that red blood cells infected with mature intracellular forms of the malaria parasite (IRBC) bind to a region located within the amino-terminal immunoglobulin-like domain of ICAM-1 that is distinct from the regions recognized by LFA-1 and rhinovirus (Ockenhouse *et al.*, *Cell 68*:63-69 (1992); and Berendt *et al.*, *Cell 68*:71-81 (1992)).

ICAM-1 has a restricted distribution *in vivo*, and its expression is regulated by LPS and the cytokines TNF, IL-1$\beta$, and interferon-gamma (Dustin *et al.*, *J. Immunol. 137*:245-254 (1986); Pober *et al.*, *J. Immunol. 137*:1893-1896 (1986); Pober *et al.*, *Transplantation 50*:537 (1990)). Bacterial products and/or inflammatory mediators released at sites of local tissue injury induce ICAM-1 mRNA and protein expression in a wide variety of cells. *In vitro*, human umbilical endothelial cells induced with TNF up regulate the surface expression of ICAM-1 and support adhesion of malaria-infected erythrocytes (Berendt *et al.*, *Nature (Lond.) 341*:57-59 (1989)). *In vivo*, individuals with cerebral malaria have higher levels of plasma TNF than individuals with uncomplicated malaria or uninfected

controls. Paradoxically, an inflammatory response initiated in response to malarial infection is used to the parasites' advantage by selectively modulating the expression of receptors to which parasitized erythrocytes attach.

In principle, the receptor binding site on IRBC surfaces should be conserved and selective pressure exerted to maintain minimal structural variation unless compensatory binding to alternate receptors occur. Sequestration of malaria-infected erythrocytes to host endothelium occurs in all persons infected with the parasite regardless of clinical severity. A small percentage of infected individuals, independent of parasitemia, progress to complicated and severe forms of the disease. The precise factors and mechanisms responsible for severe malaria are unknown. While the majority of parasitized erythrocytes from naturally-acquired infections bind only to CD36 *in vitro*, a smaller subpopulation of parasitized erythrocytes from some isolates bind to ICAM-1 and CD36. IRBC bind to different receptors in different tissues depending upon the genetic regulation of host cellular receptors and the parasite cytoadherent phenotype as expressed by single or multiple counter-receptors. Deleterious effects to the host result from the sequestration of a numerically smaller proportion of IRBC expressing the pertinent counter-receptor within a population of parasitized red cells directing the binding of IRBC to capillary endothelium within the brain leading to cerebral malaria.

Antigenically diverse naturally-acquired malaria isolates demonstrate serologically defined infected erythrocyte surface epitopes. Immune sera inhibits IRBC adhesion to human umbilical vein endothelial cells in a strain-specific manner (Udeinya *et al.*, *Nature (Lond.) 303*:429-431 (1983)), and no pan-specific sera has been identified which inhibits IRBC adhesion of geographically diverse malaria isolates.

## SUMMARY OF THE INVENTION

The present invention discloses the binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes. An IRBC binds to the first $NH_2$-terminal domain of human but not mouse ICAM-1. Further, the present invention discloses that small peptides, corresponding to a contiguous sequence of ICAM-1, are capable of inhibiting the binding of an IRBC to ICAM-1. In addition, it is disclosed herein that the binding sites within domain 1 reside spatially distant from the recognition sites for LFA-1 and HRV.

A therapeutic strategy directed toward reversing parasite sequestration ultimately can protect infected individuals from the deleterious complications of vascular occlusion.

Utilizing the present invention, anti-receptor soluble ICAM-1 analogues based upon the critical contact residues for IRBC can now be engineered to bind, lyse, and kill sequestered intraerythrocytic parasites in cases of severe and complicated *falciparum* malaria, as well as diagnosis of the presence of malaria.

The two primary sites an IRBC can bind to on a non-infected cell are ICAM-1 and CD36. Therefore, the binding of an IRBC to an uninfected cell can be inhibited by providing to the cells an agent capable of binding to the ICAM-1 binding site on the IRBC, the IRBC binding site on ICAM-1, the CD36 binding site on the IRBC, or to the IRBC binding site on CD36.

By inhibiting the binding of an IRBC to a non-infected cell, the complications arising from malaria can be ameliorated.

The agents of the present invention include:

(a) agents which are capable of binding to the ICAM-1 binding site on an IRBC, said agents selected from the group consisting of ICAM-1, a fragment of ICAM-1, a functional derivative thereof, a peptide, an

antibody, or a carbohydrate;

(b) agents which are capable of binding to the IRBC binding site on ICAM-1, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate;

(c) agents which are capable of binding to the CD36 binding site on an IRBC, said agents selected from the group consisting of CD36, a fragment of CD36, a functional derivative of CD36, a peptide, an antibody, or a carbohydrate; and

(d) agents which are capable of binding to the IRBC binding site on CD36, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate.

For example, the present invention includes the peptide agent whose amino acid sequence is: GSVLVT (SEQ ID NO 1). This agent is capable of binding to the ICAM-1 binding site of an IRBC.

The invention further includes a method for producing a desired hybridoma cell that produces an antibody which is capable of binding to the IRBC binding site on ICAM-1, the ICAM-1 binding site of an IRBC, the IRBC binding site on CD36, or the CD36 binding site of an IRBC.

The invention further includes chimeric proteins comprising ICAM, or fragments thereof, or CD36, or fragments thereof, fused to an immunoglobulin or a fragment thereof. One such ICAM-1 fusion protein, herein designated F185G1, consists of soluble-ICAM-1 fused to the hinge region and constant domains CH2 and CH3 of human IgG1 heavy chain. One such CD36 fusion protein, herein designated D30F492, consists of soluble-CD36 fused to the hinge region and constant domains CH2 and CH3 of human IgG1 heavy chain.Fusion proteins of this nature have been demonstrated to stimulate phagocytosis of an IRBC when bound to the IRBC's surface.

The inv ntion further includes a method of stimulating phagocytosis of an IRBC in a patient with malaria comprising

administering to said patient a therapeutically effectiv amount of a fusion protein comprising ICAM-1, or a fragment thereof, and/or CD36, or a fragment thereof, each of which is fused to an immunoglobulin or a fragment thereof.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1.

Binding of malaria-infected erythrocytes to chimeric forms of ICAM-1. Chimeric molecules were generated as described in Staunton *et al.*, *Cell 61*:243-254 (1990) and transfected into COS cells. The two chimeric molecules are composed as follows: hmICAM-1 (human ICAM-1, domains 1 and 2; murine ICAM-1, domains 3-5) and mhICAM-1 (murine ICAM-1, domains 1 and 2; human ICAM-1, domains 3-5). Results represent the mean of three determinations ± standard deviation.

Figure 2.

Alignment of amino acids in first amino-terminal domain of human ICAM-1, murine ICAM-1, and human ICAM-2. Amino acid substitution mutations within human ICAM-1 affecting binding of *Plasmodium falciparum* IRBC (Pf), LFA-1 (L), and HRV (R) are indicated by the solid line. The alignment of sequences by predicted secondary structure is indicated by β-strands A-G.

Figure 3.

Effect of ICAM-1 peptides on IRBC binding.

A. Inhibition of binding of malaria-infected erythrocytes to ICAM-1 by overlapping synthetic hexapeptides. ItG-ICAM IRBC and ICAM-1 hexapeptides (500 ug/ml) were added to ICAM-1 coated plates for 60 minutes. Th peptides were acetylated at the N-terminus, amidated at the C-terminus. Aba is alpha amino butyric acid and is substituted in

sequence for Cys. Results represent the mean ± s.d. of three determinations and are compared to control IRBC binding to ICAM-1 in absence of peptides.

B. Dose-dependent inhibition of IRBC binding to ICAM-1 by peptides $Pro^{12}$-$Thr^{23}$ and GSVLVT and sICAM-1, ItG-ICAM-1 IRBC (closed symbols) and ItG-CD36 IRBC (open symbols) and sICAM-1 or synthetic peptides at concentrations indicated were incubated on plates previously coated with 10 ug/ml ICAM-1 or 1 ug/ml CD36, respectively. Binding of IRBC to adhesion receptors were determined and the results represent the mean per cent binding compared to control samples incubated in PBS alone. Control binding of ItG-ICAM-1 IRBC to purified ICAM-1 is 1578 ± 225 IRBC/$mm^2$ and binding of ItG-CD36 to purified CD36 is 860 ± 108 IRBC/$mm^2$.

Fig. 4.

A. Schematic diagram of the F185G1 expression construct (pCDF185G1) and the F185G1 immunoadhesion.

B. SDS-PAGE of the immunoadhesin. COS cells were transiently transfected with the plasmid pCDF185G1 or as a control CDM8 and labeled with [$^{35}$S] methionine and cysteine. Secreted material was precipitated with protein A-Sepharose and subjected to SDS-PAGE and fluorography. Identical results were obtained with immunoprecipitations with anti-ICAM-1 mAB R6.5 (data not shown).

Fig. 5.

*P. falciparum*-infected erythrocyte and T-lymphoblastoid cell biding to recombinant ICAM-1.

A. Adhesion of ItG-ICAM-1-IRBC to surfaces coated with the indicated concentrations of ICAM-1-IgG1 chimera (F185G1), CHO cell-derived soluble ICAM-1 and *baculovirus*-derived soluble ICAM-1.

B. Inhibition of IRBC adhesion to ICAM-1-coated surfaces by F185G1 chimera, sICAM-1, or human IgG.

C. Binding of T-lymphoblastoid cells + or - PMA to F185G1 coated surfaces.

D. Inhibition of PMA-stimulated SKW-3 adhesion to sICAM-1-coated surface by F185G1.

Fig. 6.

Phagocytosis of *Plasmodium falciparum*-infected erythrocytes by human monocytes.

Fig. 7.

Monocyte phagocytosis of *Plasmodium falciparum*-infected IRBC.

A. CD36-binding IRBC preincubated with F185G1 chimera bind to the monocyte surface but are not phagocytosed.

B and C. ICAM-1-binding IRBC pr incubated with F185G1 chimera are phagocytosed and internally degraded by monocytes.

D. ICAM-1-binding IRBC in the absence of ICAM-1 immunoadhesin are not phagocytosed by monocytes.

Conditions for F185G1 mediated IRBC phagocytosis were as described in the Example for Figure 6.

Fig. 8.

Amino acid sequence of CD36. The residue used in generating the chimeras are identified in the sequence.

## DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention is based on the identification of the two primary binding sites an IRBC can bind to on a non-infected cell. These sites are contained on ICAM-1 and CD36. The present invention discloses that the binding site on ICAM-1 for *Plasmodium falciparum*-infected erythrocytes is the first $NH_2$-terminal domain between residues $Gly^{14}$-$Ser^{22}$ of human, but not mouse, ICAM-1. Further, it is disclosed herein that a peptides with an amino acid sequence selected from this region, can block the binding of an IRBC to ICAM-1.

Utilizing the amino acid sequence of the binding site, the present invention provides agents and methods for the treatment and diagnosis of malaria.

### I. Agents of the Present Invention

The present invention includes:

(a) agents which are capable of binding to the ICAM-1 binding site on an IRBC, said agents selected from the group consisting of ICAM-1, a fragment of ICAM-1, a peptide, an antibody, or a carbohydrate;

(b) agents which are capable of binding to the IRBC binding

site on ICAM-1, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate;

(c) agents which are capable of binding to the CD36 binding site on an IRBC, said agents selected from the group consisting of CD36, a fragment of CD36, a peptide, an antibody, or a carbohydrate; and

(d) agents which are capable of binding to the IRBC binding site on CD36, said agents selected from the group consisting of a peptide, an antibody, or a carbohydrate.

(e) agents which are capable of stimulating phagocytosis of an IRBC, said agents selected from the group consisting of an immunoglobulin, or fragment thereof, fused to ICAM-1, a fragment thereof, CD36, or a fragment thereof.

These agents are capable of blocking the binding of an IRBC to either ICAM-1 or CD36.

In addition, the present invention includes functional derivatives of the above described agents.

As used herein, a "functional derivative" of an agent of the present invention is an agent which possesses a biological activity that is substantially similar to the biological activity of the agent it is a derivative of. For example, if the agent is capable of binding to the ICAM-1 binding site of an IRBC, then the functional derivative will possess this binding ability. The term "functional derivative" includes "fragments," "variants," and "chimeras" of the parent molecule.

A "fragment" of an agent is meant to refer to any subset of the agent it is derived from. Fragments of ICAM-1 or CD36 which contain IRBC binding activity and are soluble are especially preferred. Soluble fragments of CD36 or ICAM-1 can be rationally designed by one skilled in the art. Generally, soluble fragments are generated by deleting the trans membrane regions of the molecule. Additionally, some of the more hydrophobic regions of the protein can b deleted.